

Investigations into the Fibrinolytic System and  
Haptoglobin Types in Certain Hereditary and Acquired  
Haemostatic Disorders and the Presence of Circulating  
Anticoagulants in Such States.

by

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"My first article of belief is based on the observation,  
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INTRODUCTION"A" The Fibrinolytic System

The fact that shed blood clots has been known to man since time immemorial and he has been aware of its protective action in the presence of those noxious stimuli which cause bleeding. Thousands of years ago, the physicians of Ancient Egypt preached the value of the application of raw meat to fresh wounds (Ghalioungui, 1961). More recently tissue extracts have been found to contain an essential factor in the extrinsic system of blood coagulation, so that when added to plasma they cause the latter to clot, a property on which the well known test, the Quick one stage prothrombin time is based (Quick, 1935).

It has only been appreciated more recently, however, that ultimately a solid blood clot normally undergoes 'dissolution' and 'liquefaction'.

The observation that, after sudden death of a person the blood clots and liquefies spontaneously afterwards, was first made more than 200 years ago by the Italian anatomist and pathologist Giovanni Bathista Morgagni (1761) and some years later, the famous English surgeon and anatomist John Hunter (1794) reaffirmed this fact. Other important historical findings on the subject followed such as, the time for lysis of the clot (Denis, 1838), non-coagulability of fibrin once it had liquefied (Green, 1887), and the role of chloroform, ether and thymol in the activation of 'fibrinolysis' (Denys and Marbaix, 1889). In 1893, the process of lysis of a blood clot was firmly established as an entity by the French biochemist Dastre who coined the term 'fibrinolysis' for it and by 1903, Hedin had demonstrated that the factor responsible for fibrinolysis resided in the globulin fraction of serum.

The experimental production of an in vivo fibrinolysis by peptone injections in dogs was achieved in 1905 by Nolf. It was not, however,

until 1933 after Tillet and Garner showed that extracts of beta haemolytic streptococci caused the lysis of plasma clots that the new field for the application of fibrinolysis in the treatment of thromboembolic conditions could be considered.

A few years later, Yudin in Russia (1936) started to use cadaver blood for transfusions, selecting persons who died from sudden accidents since these were (a) previously healthy and (b) because their blood underwent rapid liquefaction and thus could be used without anticoagulants.

One of the major contributions to our modern knowledge on fibrinolysis stems from the work of Macfarlane begun some 25 years ago by the investigation of fibrinolysis in patients who had undergone surgical operations (Macfarlane, 1937). This work, in which for the first time methods were presented for measuring the degree of fibrinolysis, opened the way to the study of the implications of fibrinolysis in various clinical states. Despite the great amount of investigation which followed, so that now the literature on the subject is voluminous, our knowledge on the basic stages of the process is still not unequivocally established and much work remains to be done. In fact, even one of the main potential applications of fibrinolysis in clinical medicine which is the use of some of its components or activators for inducing therapeutic thrombolysis still remains in the experimental stage.

Added to the lack of knowledge about fibrinolysis is the confusion that exists about the subject. This has been caused by the fact that different investigators have given many names to alleged components of the fibrinolytic system with the result that different appellations have often been given to the same constituent and sometimes the constituents on further investigation have proved to be artefacts. This confusion stresses the need for an agreement on the identity and names of the components

of the system so that an International Nomenclature can be established.

(1) Study of the Fibrinolytic System in Hereditary and  
Congenital Coagulation Defects.

The association of classical haemophilia (haemophilia due to reduction of factor VIII) with other coagulation disorders has been described not infrequently in the literature. Factor VIII deficiency has been reported in combination with deficiency of factor V (Seibert, Margolius and Ratnoff, 1958), of factor VII (Gaston, Mach and Beck, 1961), of factor IX (Sjölín, 1957) and of factor XI (plasma thromboplastin antecedent, P.T.A.) (Scardigli and Guidi, 1956). Deficiency of factor VIII has also been reported in association with a congenital vascular defect and platelet abnormality (Raccuglia and Neel, 1960) and with an ill defined freezing/serum factor (Sjölín, 1959). The presence of reduced factor VIII levels in some cases of von Willebrand's disease (Nilsson and Blombäck, 1959), is now well known to all workers in the field of coagulation disorders. There have only been a few reports of cases where the fibrinolytic system has been studied in relation to haemophilia and in these, the results have been inconsistent (Tagnon, Davidson and Taylor, 1943; Lewis, Davidson, Soulier, Tagnon and Taylor, 1946; Guest, Daly, Ware and Seegers, 1948; Richert, 1949).

Because there have been few reports of the fibrinolytic system in hereditary and familial haemorrhagic disorders, our knowledge on the matter is scanty. Experimentally, however, it has been customary to regard the level of factor VIII and the degree of fibrinolytic activity as being related in an inverse manner that is, the level of factor VIII diminishes when there is an increase in fibrinolytic activity. This is demonstrated in therapeutic trials of fibrinolytic agents, where factors I (fibrinogen),



V and VIII have been shown to decrease after the administration of large doses of fibrinolysins. These three factors have very similar physico-chemical properties and behave oppositely to the 'prothrombin group' of factors, namely II (prothrombin), VII, IX and X all of which remain unaffected (Koller, 1961). Stefanini and Gendel (1953) report, however, that increased fibrinolysis may cause excessive destruction of all known coagulation factors. In spontaneous crises and post-operatively in haemophilia where the level of factor VIII (A.H.G.) may decrease, it is not known whether this decrease is a primary one, or else secondarily aggravated by the presence of a potent fibrinolysin (Davies, 1961).

The investigation of the fibrinolytic system in haemophilia and in congenital and hereditary haemorrhagic disorders appeared to be especially useful as a means of evaluating the theory that haemostasis is a continuous process in a state of dynamic equilibrium. The theory implies continuous deposition of fibrin on the vascular endothelial lining and its balanced removal under two opposing forces of coagulation and fibrinolysis. It was thought that if the theory held true, a disturbance of one of the processes, as in haemophilia and some other bleeding disorders, would be followed by a similar disturbance in the opposing process, that is in fibrinolysis. The subject will be discussed in more detail later.

(2) Study of the Changes in the Fibrinolytic System During Extracorporeal Circulation for Open-Heart Surgery.

During extracorporeal circulation for open-heart surgery, bleeding tendencies have been reported and there has been a wide diversity of opinion about their aetiology (see p. 61). An excessive activation of



the fibrinolytic system has been more frequently reported than any other cause, and in some cases has lead to serious bleeding episodes. The cause of the sudden activation of the fibrinolytic mechanism has been the subject of much speculation. Many factors have been blamed including the type of apparatus used, the duration of perfusion, active trauma, massive transfusion, blood incompatibility, inadequate cleaning of equipment, the type of cardiac lesion and especially thromboplastin activation. Agreement, however, on the changes occurring in factors involved in the fibrinolytic and coagulation systems during extracorporeal bypass is far from unanimous.

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Because of these controversies concerning the fibrinolytic system in hereditary and congenital haemorrhagic disorders and during extracorporeal circulation, it was decided to investigate the changes occurring in this system with the aims of a.) improving the assay methods for various components of the system. b) studying the various components of the fibrinolytic system in congenital and hereditary haemorrhagic diseases, to determine the behaviour of these with operative procedures and above all to evaluate the theory of continuous fibrin formation and 'dissolution'. c.) determining the changes occurring during cardiopulmonary bypass using the Melrose N.E.P. oxygenator and heart-lung machine with hypothermia.

"B". Haptoglobin Types in Scotland and in Scottish Haemophilics.

Polonovski and Jayle in 1939 found that the peroxidase activity of haemoglobin of different species was greatly increased when plasma was added to it, and this phenomenon was shown to vary with pathological states. The same authors demonstrated that the responsible agent for

this activity resided in the proteins of the plasma. These have been recently isolated and called haptoglobins (Hp) because of their interaction with haemoglobin (Hb) (Laurell and Nyman, 1957) and their chemical and physical properties have been studied (Jayle and Boussier, 1954; Guinand, Tonnelat, Boussier and Jayle, 1956).

In 1955 Smithies demonstrated that there are three types of haptoglobins. These are now known to be genetically determined by a pair of autosomal allelic genes. Haptoglobin levels have been found to vary with disease entities though no definite relationship has been found between haptoglobin types, transferrin types and various diseases. Reports on such associations have been contradictory (Bennett, 1961). Harris, Robson and Siniscalco (1959) consider that this work should be extended.

It was decided to study the haptoglobin phenotypes in haemophilics in comparison with normal people domiciled in the same area in an endeavour to detect possibly a genetic linkage and to throw more light on the equivocal relation between haptoglobin types and/or blood groups, and various disease conditions in general.

#### "C". Circulating Anticoagulants in Factor VIII Deficiency

There are, in Scotland, approximately 220 known sufferers from factor VIII and factor IX deficiency. Of these, it is estimated that about 110 are severely affected patients i.e. who suffer repetitive spontaneous bleeding throughout their lives. Severely affected haemophilics tend to average at least one admission per year to hospital in their childhood and early adult life, ranging from minor episodes of 2-3 weeks' duration to more major ones that may last several months (Davies, 1961).

During hospitalization, haemophilics frequently require fresh blood or plasma transfusions or antihaemophilic fraction (A.H.F.) but despite the large volume of such products used in the treatment of these patients, and the reported acquired circulating anticoagulants have not been frequent. Among 67 registered haemophilics living in South East Scotland, only one case during the last 4 years was known to develop a circulating anticoagulant which destroyed factor VIII. The patient was a severe haemophilic undergoing dental extraction. The anticoagulant occurred late in his post-operative treatment and whilst proving troublesome was not lethal.

An unusual case of another haemophilic in whom a circulating anticoagulant had developed and who was admitted to the Royal Infirmary of Edinburgh from Northern England was studied. In this patient the circulating anticoagulant was unusual in that it did not destroy factor VIII but prevented thromboplastin generation, proved refractory to treatment and the patient died. A clinical and laboratory study of the case is reported later in this thesis.

PART ONE

FIBRINOLYTIC SYSTEM STUDIES

"A"

REVIEW OF LITERATURE.

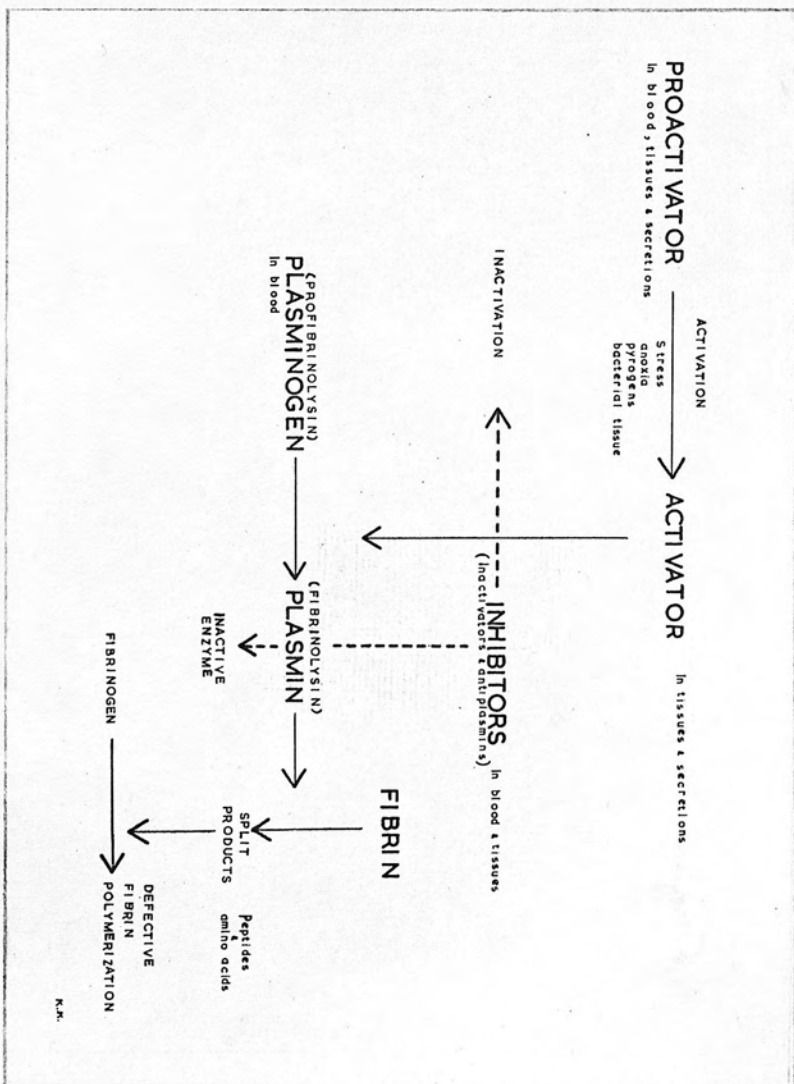


Figure 2 Plasminogen plasmin system. Hypothetical construction based on present knowledge.



### Coagulation and Fibrinolysis

The mechanism of blood clotting is a ramified process designed for the precipitation of fibrin, the fibrils of which interlace to enmesh not only the liquid plasma but also the formed elements of blood (Hoffman, 1955). This phenomenon involves the interplay of complex protein substances, most of which have not been isolated in pure form.

Our knowledge of blood coagulation can be divided into three eras by the classical theory of Morawitz postulated in 1904. These three eras are the pre-classical the classical and the post-classical. In the classical theory, prothrombin is converted to thrombin by the action of thromboplastin and calcium and the thrombin then converts fibrinogen to fibrin. Within certain limits, the Morawitz theory is still valid.

The post-classical era began in 1943 following renewed interest in, and investigations of, Quick's one stage prothrombin test and has culminated in the general recognition of the existence of twelve separate factors essential for the formation of a normal clot (Douglas, 1962). These factors have now been numbered internationally (International Nomenclature Committee, J. Amer. med. Ass., 170, 325, May, 1959) which has considerably clarified the problem in that many workers had separately described and named factors which have subsequently proved to be the same factor. These factors with their international Roman numerals and common synonyms are listed in table I.

TABLE I

International Nomenclature of Coagulation Factors

International nomenclature.	Relatively common synonyms for coagulation factors.
I	Fibrinogen
II	Prothrombin
III	Thromboplastin (tissue extract), thrombokinase.
IV	Calcium
V	Labile factor, plasma accelerator globulin, proaccelerin, plasma prothrombin conversion factor.
VI	Not used at the present time.
VII	Stable factor, serum prothrombin conversion accelerator (SPCA), proconvertin.
VIII	Antihaemophilic globulin (A.H.G.), antihaemophilic factor (A.H.F.), antihaemophilic globulin A, thromboplastinogen, platelet co-factor I.
IX	Christmas factor, plasma thromboplastin component (PTC), antihaemophilic globulin B, platelet co-factor II.
X	Stuart-Prower factor.
XI	Plasma thromboplastin antecedent (P.T.A.), Rosenthal factor.
XII	Hageman factor, contact factor.

There is no general agreement on the role of each factor in the coagulation process which results in the formation of a solid blood clot. Figure 1 shows a scheme of the interaction of the various factors participating in the clotting mechanism in which there are three stages and which normally leads to the formation of and the deposition of fibrin. Other elements which may participate in the clotting mechanism but for

which experimental proof or function is uncertain such as the erythrocyte factor, electric charge, mineral salts apart from calcium, water wettability, pH of medium, enzymes, amino acids and oxidizing agents have been omitted.

### Figure 1

#### Clot Formation and Digestion in vitro

First Stage : Formation of prothrombin activator.

a. Extrinsic System

Factor V

Factor III (tissue extract)  
or thromboplastin

Factor VII

Factor X (Stuart-Prower)

Factor IV (Calcium)

b. Intrinsic System

Activated Factor XII (contact factor or Hageman factor-activated)

Activated factor XI (P.T.A.)

Activated factor IX (Christmas factor)

Activated factor VIII (A.H.G.)

Factor IV (calcium)

Platelet factor

Factor V

Factor X (Stuart-Prower factor)

Second Stage : Thrombin generation

Factor II (prothrombin)  $\xrightarrow[\text{(Extrinsic or intrinsic)}]{\text{Prothrombin activator}}$  Thrombin

Third Stage : Fibrin formation.

Factor I (fibrinogen)  $\xrightarrow{\text{Thrombin}}$  Fibrin monomer + fibrinopeptides

Fibrin monomer  $\longrightarrow$  Fibrin polymer

Fibrin polymer  $\xrightarrow[\text{Factor IV (Calcium)}]{\text{Fibrin stabilizing factor}}$  Fibrin

A fourth stage, of no less importance than the preceding three, that of fibrin digestion or fibrinolysis, serves to remove the normal fibrin structure when it is no longer required. A proposed scheme for the

interaction of the various components of this stage of fibrinolysis is shown in figure two.

It has been claimed that coagulation and fibrinolysis that is the deposition of fibrin on, and the removal of fibrin from the vascular endothelium is in a state of dynamic equilibrium. It has been said that this equilibrium ensures the integrity of blood vessels without impairing the vitality of organs supplied by them. The assumption of such an equilibrium is an attractive hypothesis which it is one purpose of this study to evaluate on the basis of investigations into the fibrinolytic system in some cases of congenital and hereditary haemorrhagic disorders (see p 51 ).

#### Role of the Fibrinolytic System

Whilst emphasis is usually placed on the action of the fibrinolytic system within the vascular tree, it should be remembered that fibrin deposition can also occur extravascularly as in the presence of inflammation and that the fibrinolytic mechanism is therefore best considered as a generalized reparative process. The system is concerned with limiting inflammatory spread, with the healing of wounds and with maintaining the patency of duct systems in general (blood vessels, ureters, lacrymal and milk ducts etc.). It is probably involved in anaphylactic reactions (Ungar, 1947), in body protein catabolism and possibly in some manifestations of the inflammatory process such as pain, increased permeability and oedema (Biggs and Macfarlane, 1962). It is not clear whether fibrinolysis takes an active part in the clotting process itself. Recent interest in the fibrinolytic system has been stimulated by the potential use of fibrinolytic agents in the treatment of thrombotic and embolic states.

#### Nomenclature

All mammalian blood contains a significant amount of an enzyme

precursor which on activation, can rapidly hydrolyse fibrin and some other proteins. This precursor 'plasminogen' or 'profibrinolysin' in its active form is known as 'plasmin' or 'fibrinolysin' respectively, and the first terms are best used in that the active enzyme digests not only fibrin but various other proteins as well (Christensen and MacLeod, 1945). Where it is intended to restrict the action to fibrin, the terms 'fibrinolysis' and 'fibrinolytic activity' should be used. The terms 'thrombolysis' and 'thrombolytic activity' are used to describe the entire mechanism whereby thrombi or fibrinous exudates are lysed; and 'proteolysis' and 'proteolytic activity' are used to describe the overall action on proteins in general. When the blood plasmin level is high, the term 'hyperplasminaemia' can be used. It is strongly suggested that the term 'inhibitors' used by some workers as a synonym for 'circulating anticoagulants' (Nour-Eldin and Wilkinson, 1958; Israëls, Foerster and Zipursky, 1960), should be restricted to inhibitors of the fibrinolytic system which comprise both 'inactivators' and 'antiplasmins' (figure 2). Many reviews of fibrinolysis (Christensen, 1954; Astrup, 1956; Mullertz, 1956; Sherry and Alkjaersig, 1957; Sherry, Fletcher and Alkjaersig, 1959) have been published.

Variations in the Blood 'Fibrinolytic Activity'  
in Response to Physiological, Experimental and Pathological  
Conditions

Sterile clots of blood and plasma undergo various degrees of lysis after extended periods of incubation due to the spontaneous occurrence of low levels of fibrinolytic activity in normal individuals (Fearnley and Tweed, 1953; Fearnley and Lackner, 1955). The phenomenon may be accelerated by mere dilution of the plasma (Macfarlane, 1937; Macfarlane and Pilling, 1946; Bidwell, 1953; Fearnley and Tweed, 1953). A diurnal fluctuation in fibrinolytic activity has been observed by Buckell and Elliott (1959 a) and



has been confirmed in this study (see p. 48). There are suggestions that the fibrinolytic system of the newborn infant may differ from that in the adult (Quie and Wannamaker, 1960).

Besides therapeutically induced fibrinolysis which will be discussed later, various stimuli have been shown to enhance the fibrinolytic activity of blood, such as mental stress (Macfarlane and Biggs, 1946), physical exercise (Bidwell, 1953) and menstruation on the first day (Smith and Smith, 1945). The rise in fibrinolytic activity after prolonged heavy exercise is soon followed by a fall (Ogston, 1961). The injection of adrenaline causes a rise in the fibrinolytic activity (Biggs, Macfarlane and Pilling, 1947) although this effect has been questioned (Truelove, 1951). Increased fibrinolytic activity has also been described after anoxia (Tagnon, Levenson, Davidson and Taylor, 1946) and local ischaemia (Kwaan, Lo and McFadzean (1957; 1958). Sforza and Pozzi (1960) found in normal males and females breathing various gas mixtures an increase in plasma fibrinolysis which was proportional to any increase in  $\text{CO}_2$  tension or reduction in  $\text{O}_2$  tension. Excessive fibrinolytic activity following sudden anoxaemic death (Mullertz, 1952 a) had long been made use of by the Russians to obtain large volumes of blood from fresh cadavers for transfusion purposes (Yudin, 1936; Tarasov, 1960) and in countries where voluntary blood donations are scarce and professional bleeders are relatively expensive this might be an attractive proposition.

Increased fibrinolytic activity in vivo has been found following large doses of intravenous acetylcholine by Soulier and Koupernik (1948), after intravenous pyrogens by von Kaulla (1958), after nicotinic acid by Weiner, Redisch and Steele (1958) and following the administration of a fungal agent, Aspergillin O by Karaca, Stefanini and Mele (1962). Fantl and Simon (1958) reported increased fibrinolytic activity in patients after electric

shock therapy and Cliffton (1952) observed the same phenomenon with anaphylactic shock, X-ray therapy, and paradoxically after A.C.T.H. and cortisone administration since A.C.T.H., together with fresh blood transfusion and fibrinogen, has been recommended for the treatment of fibrinolytic states by Stefanini and Dameshek (1955) and by Biggs and Macfarlane (1962).

Tissue damage is, however, the best known cause of activation of the fibrinolytic system. This is illustrated by the enhanced fibrinolytic activity in burns, during various operative manipulations and in shock (Tagnon, Levenson, Davidson and Taylor, 1946; Sforza and Pozzi, 1960 a) and especially following operations on the lungs, (Baumann, 1951), the prostate (Rollason and Shillitoe, 1954), in cranial injuries (Sforza and Pozzi, 1960 b) and in some obstetrical and gynaecological conditions (Reid, Weiner, Roby and Diamond, 1953; Ratnoff, Pritchard and Colopy, 1955). An increased activity has also been found in various disease states in particular in, liver and spleen diseases (Kwaan and McFadzean, 1956; Kwaan, McFadzean and Cook, 1957; de Nicola and Soardi, 1958), in certain haematological disorders such as polycythaemia vera (Björkman, Laurell and Nilsson, 1956), leukaemia (Cooperberg and Neiman, 1955) and multiple myelomatosis (Sirridge, Bowman and Garber, 1958) and in the course of some neoplastic diseases (Brown, Campbell and Thompson, 1962).

A decrease in fibrinolytic activity has been reported to occur physiologically in late pregnancy (Biezenski and Moore, 1958) and with alimentary lipaemia (Buckell and Elliott, 1959). Pathologically fibrinolytic activity is decreased in congestive heart failure and atherosclerosis (Sawyer, Fletcher, Alkjaersig and Sherry, 1960) and in hyaline membrane disease (Lieberman, 1961).

Mullertz (1956; 1957) showed that small amounts of plasminogen activator exist in the normal circulation and the normal fibrinolytic activity of the blood may be due to it and not to the free active enzyme, plasmin. This principle may also be true in the increased response to physiological and pathological stimuli (Sherry, Lindemeyer, Fletcher and Alkjaersig, 1959). This increased activity is thrombolytic or fibrinolytic and not fibrinogenolytic (Bidwell, 1953; Fantl and Simon, 1958) or esterolytic (Schultz, Moorman, Matoush and Lincoln, 1957) and the process is independent of the proteolytic activity of the plasma (Ratnoff, 1948). All these features are characteristic of an activator action and not of free plasmin. Sawyer, Fletcher, Alkjaersig and Sherry (1960) showed that the degree of thrombolytic activity is determined by the level of plasminogen activator. This is supported by the fact that the increased activity during physiological stimuli can be sharply depressed by the action of epsilon amino caproic acid (E.A.C.A.) - an inhibitor of the plasminogen activator - when given in doses insufficient to reduce plasmin activity and also by the fact that there is no definite proof that reduction in antiplasmin levels has ever resulted in an enhanced fibrinolytic activity (Sherry, Fletcher and Alkjaersig (1959). Kwaan, Lo and MacFadzean (1957; 1958; 1958 a), studying fibrinolytic activity in vivo have produced evidence of the existence of a cholinergic effector mechanism in vessel walls which responds to ischaemia (locally and reflexly) by the release of activator into the circulation. Sherry, Lindemeyer, Fletcher and Alkjaersig (1959) studied the proteolytic activity of blood after electric shock, pyrogen injection and sever exercise and found an increase in its activity to be due to the presence of a plasminogen activator but they failed to find

any significant changes in plasmin, plasminogen, antiplasmin or fibrinogen.

There would appear to be at least two possible mechanisms causing uncontrollable fibrinolysis (hyperplasminaemia), the first causing the release of excessive quantities of activator from the tissues and the second to be due to the release of thromboplastic material which thereby results in a secondary fibrinolytic state. The clinical aspect is often too complex for present techniques to solve.

#### Bleeding Due to Excessive Fibrinolysis

The fibrinolytic haemorrhagic states seen during and immediately following operation and rarely non-surgical are sudden and unpredictable in onset. The bleeding endangers life, fibrinogen being completely absent in the circulating blood. The condition is transitory and is treated by transfusion of fresh blood and the administration of A.C.T.H and fibrinogen (Stefanini and Dameshek, 1955).

#### Plasminogen

Plasminogen (profibrinolysin) is the inactive precursor of the active enzyme plasmin or fibrinolysin. Plasminogen present in the plasma has the characteristics of a gamma globulin (Lewis, Walters, Didisheim and Merchant, 1958). The suggestion of Barnhart and Riddle (1961) that eosinophils might synthesize and transport plasminogen is yet to be confirmed. Plasminogen may be precipitated from plasma or serum at pH 5.3 and low ionic strength (Sherry, Fletcher and Alkjaersig, 1959). Remmert and Cohen (1949), Kline (1953) and Fletcher (1954) have described methods for the purification of plasminogen from plasma; and recently plasminogen has been isolated from Cohn fraction III with



an increase in specific activity of about 400 times compared to that of serum (Kline and Fishman, 1961 a).

The physico-chemical properties of plasminogen have been studied by Shulman, Alkjaersig and Sherry (1958) and the physical properties by Davies and Englert (1960). A calculated molecular weight of 83,800 for highly purified human plasminogen has been reported by the latter investigators. Purified plasminogen is sparingly soluble at neutral pH, but is soluble below pH 4.0 and above pH 8.6. There are indications that plasminogen may be a glycoprotein since plasminogen preparations contain about 1% trichloroacetic acid - insoluble hexose, part of which is rendered trichloroacetic acid - soluble during its activation (Alkjaersig, Fletcher and Sherry, 1958; 1958 a; Shulman, Alkjaersig and Sherry, 1958). Phosphorus seems to be a constituent element of the plasminogen molecule because it remains even after prolonged dialysis (Shulman, Alkjaersig and Sherry, 1958).

Plasminogen has a tremendous affinity for fibrinogen and for fibrin. Fibrinogen and fibrin preparations free from adsorbed plasminogen are difficult to obtain unless special methods are used to denature plasminogen (Lassen, 1952) as for example the heating process in the preparation of fibrin plates. On the other hand, separation of plasminogen from fibrinogen does not present a problem. In various exudates and transudates, a correlation exists between plasminogen and fibrinogen levels; where fibrinogen is low or absent plasminogen is reduced or absent and vice versa (Christensen, 1954). The biochemical basis for the affinity between plasminogen and fibrinogen or fibrin has not been determined.

Differences have been reported between the plasminogen of different species, and starch-gel electrophoresis in acid medium of bovine and human



plasminogen gave different patterns (Cole and Mertz, 1961 a).

Stability of plasminogen: (Christensen and Smith, 1950; Troll and Sherry, 1955; Ablondi and Hagan, 1957; Lassen, 1958).

Plasma plasminogen appears to be stable at moderate temperatures over a wide pH range. Purified plasminogen preparations are, however, relatively unstable at neutral and alkaline pH. Below pH 4.0, purified plasminogen is quite stable and may be brought to 100°C. at pH 2.0 without significant loss of activity (Sherry, 1954; Troll and Sherry, 1955).

#### Proactivator

The nature of a hypothetical serum and plasma proactivator has been the subject of considerable controversy. The hypothetical basis for the presence of such an activator is based on observations of the varying behaviour of streptokinase towards activation of plasminogen in different species. Streptokinase (SK) is a bacterial protein which transforms inactive plasminogen into active plasmin. SK which readily activates human plasminogen has almost no action on bovine and porcine plasminogen (Cliffon and Downie, 1950), unless small quantities of human serum are added (Mullertz and Lassen, 1953; Sherry, 1954; Troll and Sherry, 1955). It has been deduced, therefore, that human serum contains a substance termed proactivator (Mullertz and Lassen, 1953; Astrup, 1956 a; Mullertz, 1956) which is absent in bovine and porcine sera. Further, the activation of human plasminogen by SK takes place in a two-step manner. SK first reacts stoichiometrically and perhaps reversibly with a serum factor to produce an activator. This activator then enzymatically activates plasminogen by first order kinetics (Mullertz and Lassen, 1953; Mullertz, 1955; Troll and Sherry, 1955; Sherry and Alkjaersig, 1957; Alkjaersig, Fletcher and Sherry, 1958 a).

Differential stability studies lend support to the existence of such a proactivator (Troll and Sherry, 1955; Mullertz 1957; Lassen, 1958).

The main confusion regarding the nature of proactivator is whether it is linked in some way to human plasminogen or is a distinct entity (Troll and Sherry, 1955; Ablondi and Hagan, 1957; Kline and Fishman, 1957; Mullertz, 1957; Sherry and Alkjaersig, 1957; Baumgarten, Cole, Richard and Smith, 1958). Evidence associating proactivator activity with human plasminogen has been produced (Sherry, 1954; Ablondi and Hagan, 1957; Kline and Fishman, 1957; Sherry and Alkjaersig, 1957; Hagan, Ablondi and De Renzo, 1960) and in all purification techniques so far employed, the proactivator activity parallels exactly that of the human plasminogen content of the purified fractions. Lassen (1958) has argued, however, that plasminogen cannot be identical with proactivator since during the conversion of the latter to activator, the plasminogen and plasmin concentrations remain unchanged.

Kline and Fishman (1961) have demonstrated a proactivator function of human plasmin by a lysine esterase assay.

#### Activators

Our knowledge about the activation of the plasminogen system is very incomplete since most of the factors concerned have not been identified nor has their mode of action been determined. The following classification of in vitro activators which is a modified version of Sherry, Fletcher and Alkjaersig (1959) is recommended.

##### A. Naturally occurring activators

1. Tissue activators.
2. Activators in plasma and other biological fluids.
3. Urinary activators.

4. Trypsin.
5. Plasmin.
6. Phosphatases.

B. Activators of bacterial origin

1. Streptokinase (SK)
2. Staphylokinase.

C. Miscellaneous activators.

1. Chloroform and other organic solvents.
2. Peptone.
3. Urea and aromatic derivatives; hydrotropic substances.

A. Naturally Occurring Activators:

1. Tissue activators. Tissue kinases or fibrinokinases.

Various animal tissues have been shown to possess an activating action on the plasminogen system (Astrup and Permin, 1947; Permin, 1947; Astrup, 1951). These tissue activators can be extracted in soluble form by concentrated potassium thiocyanate solutions (Astrup and Sterndorff, 1956; 1956 a). Different tissues vary considerably in their activator content (Albrechtsen, 1957; 1957 a; Roberts and Astrup, 1957; Astrup and Sjölin, 1958); in man, lung and brain have the highest concentration (Cohn and Warren, 1961) and the prostate, the thyroid and the uterus also have specially high concentrations (McNicol, 1962).

Todd (1959) described a histological technique for the detection of fibrinolytic activity in tissues. His results show that fibrinolytic activators in human tissues are concentrated principally in the endothelium of veins, venules and the pulmonary arteries. It has been claimed that the adventitia in the aorta has a high concentration of activators, whereas the intima and media contain thromboplastin (Cohen and Warren, 1961).

The presence of large amounts of activators of the plasminogen system in many tissues and the close affinity of plasminogen for fibrin, lends support to the concept that fibrinolysis via the plasminogen system is of major importance in the resolution of inflammatory and traumatic fibrinous exudate.

Tissue activators are fairly thermostable particularly at acid pH, but gross impurities in the preparations have prevented further identification and it remains to be established whether the activators produced by different tissues are similar (Sherry, Fletcher and Alkjaersig, 1959).

2. Activators in plasma and other biological fluids. Trace amounts of activators may be present in the circulation of normal individuals (Mullertz, 1956; 1957). Activators have also been detected in bodily secretions such as human milk (Astrup and Sterndorff, 1953), tears (Storm, 1955), saliva (Albrechtsen and Thaysen, 1955) and seminal fluid (von Kaulla and Shettles, 1953). Activators have also been demonstrated in patients after acute anoxaemic episodes (Mullertz, 1953), amniotic fluid embolism (Albrechtsen, Storm and Trolle, 1955), electric shock, pyrogen administration, exercise, ischaemia and epinephrine (Mullertz, 1953). Increased fibrinolytic activity in response to various physiological and pathological stimuli is due to the release of activators (see p. 16).

The activity of activators is almost wholly precipitated with the plasma euglobulin fraction (Mullertz, 1956; 1957). Like most of the coagulation factors and fibrinolysins, however, they have never been isolated in pure state for further characterization. Activator activity in the plasma is much less stable than are activators in tissues; its instability may be related to various inhibitors in the blood (Douglas,

1962). During the clotting process plasma activators are partially adsorbed on to the fibrin coagulum and it is by this local means, and not in the general circulation that they act on the fibrin.

3. Urinary activators. The urinary activator of plasminogen has been called urokinase by Sobel, Mohler, Jones, Dowdy and Guest (1952). In normal individuals urokinase concentrations are similar in male and female urine (Bjerrehuus, 1952), independent of age and urine volume, but alter in diseases (Sherry, Fletcher and Alkjaersig, 1959).

Astrup and Sterndorff (1952), suggest that urokinase may not be a simple excretion product but that it is locally produced in the kidneys in order to maintain the patency of the urinary system. Recent investigations suggest that urokinase excretion may represent, at least in part, excreted plasminogen activator (McNicol, Fletcher and Sherry, 1960); von Kaulla and Swan (1958) report an increased excretion of urokinase in patients who developed fibrinolytic states during cardiac surgery, returning to normal levels as the intensity of the fibrinolytic state declined.

Urokinase itself seems to be capable of protein digestion. In high concentrations it acts in varying degrees on casein, the basic amino acid esters tosyl arginine methyl ester and lysine ethyl ester, and on a heparin-protamine complex (Kjeldgaard and Ploug, 1957; Sherry and Alkjaersig, 1957; Alkjaersig, Fletcher and Sherry, 1958 a).

Urokinase forms 37% of the total urinary proteins as shown by electrophoretic studies (Sherry, Fletcher and Alkjaersig, 1959). Urokinase is non dialyzable, active at neutral pH, and although more stable than the activator in milk, tears and saliva, it is labile in acid solutions (Cohen and Warren, 1961). It is heat stable up to 50°C. and stable over



a wide pH range (Ploug and Kjeldgaard, 1957).

4. Trypsin. Trypsin is a plasminogen activator (Kocholaty, Ellis and Jensen, 1952; Alkjaersig, Fletcher and Sherry, 1958 a) but acts relatively slowly as compared to the specific activators (SK and urokinase) and differs from them in that it produces a higher percentage of trichloroacetic acid-soluble fragments and its action is not competitively inhibited by epsilon amino caproic acid in contradistinction to the action of the specific activators (Sherry, Fletcher and Alkjaersig, 1959).

5. Plasmin. Purified preparations of plasminogen undergo spontaneous activation at neutral pH but the yield of plasmin under these circumstances is poor due to the instability of both plasmin and its precursor under these conditions. This activation may be autocatalytic for it is known that plasmin when added to activator accelerates its action whereas plasmin substrates inhibit it. The activation of plasminogen by plasmin closely resembles that observed with trypsin.

6. Phosphatases. Claims that phosphatases are the main activators of plasminogen with subsequent major effects on the fibrinolytic system (Misirlioglu and Lillehei, 1962) remain to be decided.

#### B. Activators of Bacterial Origin.

1. Streptokinase (SK). In 1933 Tillett and Garner in New York discovered that rapid lysis occurred in plasma clots incubated with extracts of beta haemolytic streptococci. This is now known to be due to the bacterial enzyme streptokinase. Streptokinase is produced by actively growing haemolytic streptococci of the Lancefield's groups A, human C and G (Tillett, 1952), is readily soluble at pH's both acid and alkaline to the isoelectric point (4.7-5.0) and is stable over a wide pH range. At a pH of more than 9.0, however, it undergoes irreversible inactivation.

The action of SK appears to be restricted to plasminogen activation. It is not itself proteolytic for casein nor does it act on synthetic substrates (Sherry and Alkjaersig, 1957). However, the activator formed by the interaction of SK and the human serum factor seems to be a proteolytic enzyme (Alkjaersig, Fletcher and Sherry, 1958 a). A potent preparation of streptokinase-streptodornase "Varidase" is now marketed.

2. Staphylokinase. Strains of staphylococci vary in their production of this enzyme. Staphylokinase differs from SK because it can activate the plasminogen of many animal species (Gerheim and Ferguson, 1949; Cliffton and Cannamela, 1951; 1953).

C. Miscellaneous activators:

1. Chloroform and other organic solvents. These were the earliest known activators of the fibrinolytic system. In 1889 Denys and Marbaix found that a thermolabile proteolytic agent developed in serum after the addition of chloroform, ether or thymol. They concluded that a proteolytic enzyme present in serum was normally inhibited by some other substance which chloroform removed or destroyed and Christensen (1946) agreed with these findings. Chloroform activation is a slow process (Baumgarten, Cole, Richard and Smith, 1957) which only occurs within a narrow range of its concentration since it may otherwise denature plasminogen.

2. Peptone. Nolf (1905) produced an increased fibrinolytic activity in hepatectomized dogs by the intravenous injection of peptone. It has been recently shown that the treatment of guinea pig serum by peptone produces a plasminogen activator and also a proteolytic enzyme in the euglobulin fraction (Ungar and Mist, 1949). Olesen (1957) using the fibrin plate method obtained further evidence of the activator action

of peptone, and Astrup and Olesen (1957) partially purified the factor responsible for this action.

3. Urea and aromatic derivatives and hydrotropic substances. von Kaulla and Smith (1961) have observed that activity similar to that exerted by SK can be brought about by certain urea derivatives (but not by urea itself). von Kaulla (1962) later found that hydrotropy is a common denominator of the active urea derivatives and of some other aromatic compounds which activate the fibrinolytic system in vitro.

#### Mechanism of Plasminogen Activation.

In their comprehensive review on fibrinolysis, Sherry, Fletcher and Alkjaersig (1959) state that "It has been demonstrated that the kinetics of activation of plasminogen by urokinase, streptokinase and trypsin are consistent with a first order enzymatic reaction (Alkjaersig, Fletcher and Sherry, 1958 a). Certain features are common to all these activations, including the autocatalytic activation of plasminogen: 1) significant quantities of acid-soluble nitrogen, tyrosine and carbohydrate are released from plasminogen during the activation; 2) the activation is mediated by an enzyme capable of splitting arginine and lysine esters; and 3) the latter act as competitive inhibitors to plasminogen activation. Thus the activation process appears to include a proteolytic step in which lysine and/or arginine bonds are split. However, the existence of two general types of activation is suggested by the observation that differences exist between the activation occurring in the presence of certain activators (urokinase and streptokinase), and that produced by other activators (trypsin and plasmin). The former activation occurs in the presence of plasmin inhibitors, is rapid, and results in a plasmin apparently physico-chemically distinct from that obtained with trypsin or plasmin,

where activation is relatively slow and readily influenced by the presence of plasmin inhibitors (Alkjaersig, Fletcher and Sherry, 1958; 1958 a)".

#### Plasmin.

Plasmin is the active proteolytic enzyme produced by the action of activator(s) on plasminogen. Plasma, serum and possibly platelets carry antiplasmins (Johnson and Schneider, 1953; Stefanini and Murphy, 1956; Sherry and Alkjaersig, 1957) normally in a considerable excess, and provided, as is the case under physiological circumstances, plasmin formation does not take place more quickly than plasmin neutralization, no free plasmin appears in the circulation (McNicol, 1962).

Plasmin is most active at neutral pH but its isolation and purification has been difficult because of its instability at this pH. The present progress towards the preparation of pure plasminogen is essential as a means of preparing pure plasmin. At present, production of plasmin has been from plasminogen preparations either spontaneously in the presence of stabilizing agents like glycerol-buffer (Alkjaersig, Fletcher and Sherry, 1958), or by the use of specific activators, such as SK. Using the latter method Troll and Sherry (1955) activated plasminogen with SK and precipitated the enzyme at pH 2.0. Fishman and Kline (1956) carried the process a step further by alcohol fractionation of the product of SK activation. It is now, however, thought that the activity ascribed to plasmin in these preparations is probably due to the contaminating activators. Physico-chemical studies suggest that the sedimentation constant and molecular size of plasmin depend on the mode of activation (Shulman, Alkjaersig and Sherry, 1958).

Plasmin hydrolyses such naturally occurring proteins as fibrin, fibrinogen, factors V and VIII (Ferguson, 1949; Johnson, Seegers and Braden, 1952; Sherry, Titchener, Gottesman, Wasserman and Troll, 1954;



Soulier, Alagille and Larieu, 1956; Cliffton, 1957; Coon and Duff, 1958; Koller, 1961), some of the components of complement (Alagille and Soulier, 1956), A.C.T.H., growth hormone and glucagon (Mirsky, Perisutti and Davis, 1959). Other protein substrates for plasmin are casein (Remmert and Cohen, 1949; Christensen, 1954 a) gelatin (Christensen, 1945), beta lactoglobulin (Christensen, 1954) and protamine complexes (Kjeldgaard and Ploug, 1957).

There is no parallel correlation between the fibrinolytic, the fibrinogenolytic and other proteolytic activities of plasmin. Ratnoff (1953; 1955) has noted that whereas plasmin digests fibrin and fibrinogen at equal rates which accords with Christensen's observations (1945), he found that fibrin is more rapidly destroyed than fibrinogen during spontaneous activation in human blood and after the addition of SK. Ratnoff suggests that plasminogen activation is enhanced by the clotting mechanism, possibly through an effect of the fibrin surface. This is substantiated by the findings of Celander and Guest (1957). According to McNicol (1962), inhibitory mechanisms largely restrict the action of plasmin to the digestion of fibrin in vivo.

As early as 1887 Green made the fundamental observation that once fibrin is 'dissolved' it could not be coagulated by thrombin. But even now, the exact composition of the products of fibrin and other protein degradation resulting from the action of plasmin is not known. Plasmin, however, degrades fibrin into a number of peptides and amino acids. There is evidence that plasmin splits proteins at arginine and lysine bonds, in that synthetic esters of the basic amino acids arginine and lysine act as excellent plasmin substrates and as competitive inhibitors of the proteolytic and fibrinolytic activities of the enzyme (Troll, Sherry and Wachman, 1954).



Although similarities in the modes of action of plasmin and thrombin have been described (Seegers, 1952; Ronwin, 1956), the separate identity of these two enzymes has been established along many lines (Sherry, Fletcher and Alkjaersig, 1959).

#### Defective fibrin polymerization.

Recently, Fletcher, Alkjaersig and Sherry (1962) have shown that patients under treatment with plasminogen activators may develop a coagulation defect similar to that observed in patients who 'spontaneously' develop states of 'pathological plasma proteolytic activity' or 'hyperplasminaemia'. According to these authors, the major coagulation defect in these instances, is due to the presence of the products of proteolysis formed from fibrinogen or fibrin and circulating in the plasma. The two main features of these anomalies are an increase of thrombin time and a macroscopically apparent defect in clot appearance and character. The biochemical lesion responsible for these defects has been termed "defective fibrin polymerization" by Alkjaersig, Fletcher and Sherry (1962). It is not yet known how this phenomenon can be reconciled with the work of Niewiarowski and Kowalski (1958), Niewiarowski, Kowalski and Stachurska (1959) and Kowalski (1960) where it was held that such anticoagulant activity derived from the degradation products of fibrinogen was due to inhibitors of blood coagulation for example antithrombin VI, another product of fibrinolysis.

#### Inhibitors (antiplasmins).

The assumption of the presence in the circulation of inhibitors of fibrinolysis has been drawn from the observations of Denys and Marbaix (1889) that the proteolytic activity which develops after the addition of chloroform to serum is due to the removal or destruction of some inhibitory substance in the serum. This has been supported by Hahn who in 1897 noticed an

antitryptic property of serum.

Antiplasmins have been found in the blood of many animals as well as in that of man (Guest, Daly, Ware and Seegers 1948 a). It has been shown by Bundy and Mehl (1958) that there is no sex difference in the level of serum trypsin inhibitors. The inhibitory power of plasma is markedly in excess of its plasmin activity (Norman, 1958; McNicol, 1962). There has been much argument about the possible existence of two or more serum antiplasmins (Ratnoff, Lepow and Pillemer, 1954; Norman, 1957; 1958; Norman and Hill, 1958). Shulman (1955) isolated and partly characterized an interesting inhibitor of proteolytic activity from both urine and plasma.

Plasmin inhibitors have also been reported in many tissues (Cohen and Warren, 1961) and in platelets (Johnson and Schneider, 1953), but overall there have been very few such investigations, a fact which applies to inhibitors of all kinds.

Stefanini and Dameshek (1955) found that cortisone promptly reduced the activity of serum fibrinolysins and Ungar and Damgaard (1951) showed that A.C.T.H. and cortisone significantly increased the antifibrinolytic activity in the intact normal animal but that there was no such increase following the injection of A.C.T.H. or cortisone into splenectomized guinea pigs. The plasmin inhibiting properties of A.C.T.H. are discussed by Cohen and Warren (1961).

The level of antifibrinolysins is increased in nephrosis (Jacobsson, 1955), in pernicious anaemia and in pneumonia (Guest, Daly, Ware and Seegers, 1948), in platelet-rich plasma (Stefanini and Murphy, 1956) and in plasma following fatty meals (Greig and Runde, 1957).

Controversy surrounds the nature and number of plasma components

capable of inhibiting plasmin activity. Milstone (1941) found antiplasmin activity in the pseudoglobulin fraction and Macfarlane and Pilling (1946) found it to be in the supernatant of the euglobulin precipitate of low ionic strength and at pH 5.3. Evidence has now accumulated that inhibitors can be recovered from the albumin (Lewis, Walters, Didisheim and Merchant, 1958; Phillips and Skrodelis, 1958; Cherry, Adamsons and Phillips, 1961), beta one (Lewis et al., 1958), alpha one and alpha two globulins (Bundy and Mehl, 1958; Lewis et al., 1958; Cherry, Adamsons and Phillips, 1961). By Cohn fractionation it has been demonstrated that different plasmin inhibitors can be separated from fractions IV and V (Grob, 1949; Norman, 1958).

Inhibitors of the fibrinolytic system fall into two main categories, those acting upon (i) the activation process (inactivators) and (ii), active plasmin, which can be aptly called antiplasmins.

i. Inhibitors of the activation process: The occurrence of naturally occurring activator inhibitors has been proved (Lewis and Ferguson, 1951; Mullertz, 1956; 1957). Mullertz (1957) noted that a crude sample of bovine globulin inhibited the activation of bovine plasminogen by SK activated human globulin.

ii. Inhibitors of plasmin (antiplasmins). It has been shown by Sawyer, Fletcher, Alkjaersig and Sherry (1960) that plasmin is almost immediately inactivated when added to plasma, but due to technical difficulties of assay, many authors intending to measure antiplasmins have measured antitryptic activity instead (Christensen and Macleod, 1945; Christensen, 1946; Clifton, 1952; de Leeuw, Wright, and Morton, 1953). It is now known that antiplasmin and antitryptic activity of

plasma do not parallel one another which makes this approach unsatisfactory (Shulman, 1952; 1952 a; 1952 b).

#### Synthetic Inhibitors of the Fibrinolytic System

In the majority of reports, the differentiation between substances acting as activator inhibitors and those acting directly on plasmin (antiplasmins) has been unsatisfactory since the methods used have been incapable of distinguishing between them.

Epsilon amino caproic acid (E.A.C.A.), a structural analogue of lysine, was originally described as a plasmin inhibitor. It has now been shown that E.A.C.A. is a far more potent inhibitor of plasminogen activation than of plasmin (Ablondi, Hagan, Philips and De Renzo, 1959; Ablondi and De Renzo, 1959; Alkjaersig, Fletcher and Sherry, 1959). E.A.C.A. inhibits plasminogen activation by SK and by urokinase competitively. It inhibits trypsin and autocatalytic plasmin activation in a noncompetitive manner.

Quaternary amines, lauryl amine and E.A.C.A. which act as plasmin inhibitors at high concentrations, potentiate the action of plasmin at very low concentrations on a number of substrates (Astrup and Alkjaersig, 1951; 1952; Alkjaersig, Fletcher and Sherry, 1959). Sodium chloride, while inhibiting the activation of crude bovine plasminogen by SK and human globulin activator, does not inhibit the activation of human plasminogen (Cole and Mertz, 1961 a).

Other synthetic inhibitors include numerous organic substrates, dyes and related compounds (Geiger, 1952), heparin and similar substances (Astrup and Alkjaersig, 1952; von Kaulla and McDonald, 1958), basic amino acids (Mullertz, 1954), their polymers (Ginsburg, de Vries and Katchalski, 1952) and esters (Troll, Sherry and Wachman, 1954), methylamine and urea (Norman, 1957).



Inorganic ions (Ronwin, 1962) particularly the heavy metals (Kowalski and Latallo, 1956), will also inhibit or denature plasmin.

Trypsin inhibitors such as pancreatic trypsin inhibitor (Christensen and MacLeod, 1945), soya bean (Christensen and MacLeod, 1945; Astrup and Alkjaersig, 1952), and toxic phosphorus compounds (Mounter and Shipley, 1958) also inhibit plasmin action.

#### Use of Fibrinolytic Agents in the Management of Thromboembolic Conditions.

One of the chief interests in fibrinolysis and in fibrinolytic agents is the possible use of these latter substances in thromboembolic diseases and some inflammatory conditions which do not produce necrosis of tissues. Progress in the field has been slow, but, as Koller (1961) points out "Although fibrinolytic therapy was virtually started in 1933 with the discovery of the fibrinolytic effect of streptococci, 27 years later fibrinolytic treatment is still in a preliminary stage. However, the purification of heparin needed 20 years and only 30 years after its discovery was it used on a large scale. Therefore, there is no reason to be concerned about the relatively slow advance of our knowledge in fibrinolytic therapy and the many difficulties encountered in its realization". Many satisfactory results have been achieved but progress has been hampered by many controversial findings. In fact a depressed fibrinolytic activity in patients with thrombotic diseases, with atherosclerosis and following fatty meals (Nestel, 1960; Tillman, O'Neal, Thomas and Hixon, 1960) which have formed bases for thrombolytic treatment, has been denied by Hougie and Ayers (1960), Blix (1961) and by Berry (1962). The observations of Buckell and Elliott (1959) that there is less flexibility of the fibrinolytic system with advancing age may prove a useful means of research into this problem.



Many fibrinolytic agents have been investigated clinically. Streptokinase has been widely used (Koller, 1961). In a recent leading article (Brit.med.J., 1968, May 19th, 1962) much has been said about urokinase. The active enzyme, plasmin, has also been tried (Cliffton, 1961). Other agents include pyrogens (von Kaulla, 1958), fungal agents (Karaca, Stefanini and Mele, 1962), nicotinic acid (Weiner, Redisch and Steele, 1958), complamin which is a combination of nicotinic acid and theophylline (Koller, 1961), urea derivatives (von Kaulla and Smith, 1961) and an enzyme derived from Pseudomonas fluorescens (Adamis, 1961).

Difficulties with thrombolytic agents are met with in control of dosage. Febrile and allergic reactions to the drugs are now infrequent since purification methods have been improved. The development of antibodies towards SK after prolonged therapy and after acute haemolytic streptococcal infections is a potential hazard (Blix, 1961 a). In experimentally produced high levels of blood fibrinogen in dogs, an increased resistance of clots to lyse has been observed (Bang, Freiman and Cliffton, 1960).

Contraindications to fibrinolytic therapy include defective blood coagulation especially where there is depressed liver function and where there is a deficiency of factors V and VIII and of fibrinogen.

In general it may be said that well controlled fibrinolytic therapy is safe and produces less haemorrhages than some anticoagulants. This safety has been further ensured by the availability of E.A.C.A. which is a well tolerated drug.

#### Evaluation of the Methods of Assay of the Fibrinolytic System.

The process of fibrinolysis is complex, involving an interaction between the fibrin clot and the serum with which it is in contact. The

main participants in this reaction are plasminogen, proactivator and activators, plasmin, plasmin inhibitors and fibrinogen. The complexity of the system, the lack of knowledge concerning it and the inability to isolate the (hypothetical) constituents have all contributed to the difficulties in formulating assay methods and those in use have marked limitations. Preparations of fibrin and fibrinogen used as physiological substrates are invariably, significantly and seriously contaminated with plasminogen due to the tremendous affinity of the latter for fibrin and fibrinogen. Synthetic substrates such as arginine and lysine esters have been tried, but they lack specificity and other non-plasmin serum enzymes are capable of hydrolysing them (Troll, Sherry and Wachman, 1954). The effect of different reagents used on the fibrinolytic mechanism may produce variable and fallacious results which depend upon the method of assay used.

The "fibrinolytic activity" of plasma has been assayed on four main lines:-

I Depending on (1) its fibrinolytic properties (Loomis, George and Ryder, 1947; Astrup and Mullertz, 1952; Shulman, 1952; Fletcher, 1954 a; Fearnley, Balmforth and Fearnley, 1957) (2) its fibrinogenolytic properties (Mullertz, 1952).

These tests include clot lysis times of whole and of diluted blood (Chakrabarti and Fearnley, 1962), of whole and of diluted plasma (Macfarlane, 1937, Swan, 1961), tests based on the ability of serum to lyse normal clots (Miale, 1962), the use of fibrin and plasma plates (Astrup and Mullertz, 1952; von Kaulla and McDonald, 1958) and the euglobulin lysis time (Macfarlane and Pilling, 1946; Biggs and Macfarlane, 1962). Tests have also been used which measure the degree and pattern of standard clot lysis by the estimation of the amount of residual clot

based on its tyrosine content (Bidwell, 1953), the use of fluorescein labelled fibrin (Pappenhagen, Koppel and Olwin, 1962), of isotopically labelled human plasma clots (Sawyer, Fletcher, Alkjaersig and Sherry, 1960) and by thrombelastography (Hartert, 1951; de Nicola and Mazzetti, 1955; von Kaulla, 1957).

II. Based on its proteolytic properties. Substances used have included gelatin, azocoll, protamine complexes, urea-denatured haemoglobin and chiefly casein (Remmert and Cohen, 1949; Christensen, 1954 a; Kaplan, 1954; Kline and Fishman, 1961).

III. Utilisation of synthetic substrates such as lysine and arginine esters (Schultz, Moorman, Matoush and Lincoln, 1957; Lassen, 1958; Roberts, 1958)

IV. Immunochemical analysis (Hagan, Ablondi and De Renzo, 1960).

The simplest way to measure fibrinolytic activity is by the observation of the whole blood clot lysis time. Somewhat more complex but similar are methods determining, 1) the diluted whole blood clot lysis time (Chakrabarti and Fearnley, 1962), the whole plasma and diluted plasma clot lysis times (Macfarlane, 1937, Swan, 1961) and the ability of serum to lyse normal clots (Miale, 1962). These methods are valuable for detecting markedly enhanced fibrinolytic activity, but are inadequate for measuring low levels of such activity often because of the very long lysis times. The end point was once not reached for more than ten days (Swan, 1961). In addition, dilution may artificially create 'fibrinolytic activity' since Fearnley and Ferguson (1958) claim that an undiluted clot does not liquefy.

#### Fibrin and Plasma plates.

The fibrin plate test has been widely used (Astrup and Mullertz, 1952; von Kaulla and McDonald, 1958) whereby either a fibrinogen

solution or plasma is clotted in a Petri dish, inoculated with the material to be tested and incubated overnight. The area of lysis gives an estimate of 'fibrinolytic activity' of the substance tested. The fibrin plate is an excellent test for high levels (Douglas, 1962) but not for low levels of activity (von Kaulla and Schultz, 1958).

The bovine fibrin usually used as substrate contains bovine plasminogen as a contaminant and measures therefore active fibrinolytic enzymes which at the most are only present in trace amounts in plasma, as well as activators. The fibrin plate is therefore considered to measure the activator content. In order to estimate only the level of plasmin under investigation and not that due to activation of plasminogen contained in the substrate as well it has been suggested that the plasminogen content of the fibrin plate should be destroyed by heating before it is used in this test (Lassen, 1953; Mullertz and Lassen, 1953; Mullertz, 1957). Fibrin plates can also be used to assess streptokinase activity (Biggs and Macfarlane, 1962).

#### Plasma Euglobulin Lysis Time.

Macfarlane and Pilling (1946) first suggested the use of this method which is now known to be based on the fact that the plasma euglobulin (which together with some of pseudoglobulin I form the gamma globulins) (Hoffman, 1955) contain the essential clotting factors as well as plasminogen and plasmin (Cherry, Adamsons and Phillips, 1961). The plasma is first diluted so that the antiplasmins which are associated with albumin and alpha globulin fractions (Cherry, Adamsons and Phillips, 1961) remain in solution when the euglobulin fraction is precipitated by acidification to a low pH. The precipitated euglobulin is then redissolved and clotted and then invariably undergoes lysis, the time for which even under normal conditions is short enough to give a rapid result.



The method can be used even when heparin is present and will readily detect weak fibrinolytic activity. This method has the advantage of requiring neither special equipment nor a high degree of skill. It has been claimed by Buckell (1958) that the type of anticoagulant used in blood withdrawal affects the euglobulin lysis time (E.L.T.), the maximum fibrinolytic activity being found in citrated specimens, whilst specimens taken into heparin and sequestrene (E.D.T.A.) showed less activity. This illustrates the importance of standardised procedures.

The E.L.T. measures mainly the activators, the contribution by plasmin being very small. The significant, although imprecise, correlation between the isotopically - labelled human plasma clot assay (a very elaborate technique) and the plasma euglobulin lysis time technique reinforces the validity of this latter assay method (Sawyers, Fletcher, Alkjaersig and Sherry, 1960). A micro-method for E.L.T. assay suggested by Copley, Niewiarowski and Maréchal (1959) may prove to be very useful. The E.L.T. has been considered the best of the simpler methods of assay (Douglas, 1962) and has been recommended by many authors (Wintrobe, 1961; Biggs and Macfarlane, 1962) for the estimation of the "fibrinolytic activity". The use of the method in the control of thrombolytic therapy (Douglas, 1962; Poller, 1962) gives it a special importance among other tests.

#### Clot Tyrosine Method.

A method relating the lysis pattern of a series of clots incubated for varying intervals of time and measuring the amounts of residual clots by their tyrosine content has been described (Bidwell, 1953; Biggs and Macfarlane, 1962). von Kaulla and Schultz (1958) do not regard the method as a practical one from the clinical point of view and in this work the results were not found to be reproducible on duplicate assays.



### Fluorescein - Labelled Fibrin.

The technique of assay using fluorescein-labelled fibrin (Pappenhagen, Koppel and Olwin, 1962) is still in its infancy, but shows promise.

### Isotopically - Labelled Clots

In the methods using  $^{131}\text{I}$ -labelled human plasma, clots are formed from aged human plasma enriched with human plasminogen and containing trace quantities of  $^{131}\text{I}$ -labelled bovine fibrinogen. These are incubated in the material to be tested for a standard time. The residual clots are then separated by filtration and the radioactivity of the total filtrate determined. Sawyer et al., (1960), using this method found that plasma obtained from non-stressed normal adults showed activator activity which varied as a result of stress and disease. They found no sex difference, no correlation with age, no clear evidence of diurnal variation and no striking daily fluctuations in the activity among the adult population they studied. The method is too elaborate and expensive for routine use.

### Thrombelastography.

Thrombelastography as introduced by Professor Hartert of Heidelberg University (1951), permits continuous visual and kymographic observation of all phases of coagulation, including fibrinolysis. The measurement is automatic and is based on the principle of determining the elasticity of the fibrin clot. Thrombelastography can be done on intact whole blood, is rapid and reliable, even when the fibrinolytic process takes a few minutes or several hours for its completion (de Nicola and Mazzetti, 1955; von Kaulla, 1957; von Kaulla and Schultz, 1958).

### Caseinolytic Assays.

Among proteolytic assays available, the most popular is the caseinolytic one which many considered to be best routine method of estimating plasminogen (Douglas, 1962), for though plasminogen and plasmin are measured together by this method, the amount of plasmin in normal plasma is negligible. Sawyer et al., (1960), however, state that the 'thrombolytic activity' and plasma 'proteolytic activity' are independent of one another.

The method originally introduced by Remmert and Cohen (1949), has been widely adopted (Christensen, 1954 a; Kaplan, 1954; Norman, 1957). The plasma is first acidified to prevent coupling of the major portion of the plasmin with antiplasmin which would result in loss of plasminogen. The plasminogen is then converted to plasmin by SK in which form it can be measured by hydrolysis of casein and the surplus undigested casein is then precipitated with trichloroacetic acid and the trichloroacetic acid-soluble tyrosine estimated by spectrophotometry.

### Use of Synthetic Substrates.

The use of synthetic substrates by Schultz, Moorman, Matoush and Lincoln (1957) and by Roberts (1958) has proved unsatisfactory in the investigation of physiological fibrinolysis, probably because fibrin due to its strong adsorption of fibrinolytic factors, is a much more susceptible substrate and thus reacts when other substrates remain unchanged (Biggs and Macfarlane, 1962). Furthermore, these synthetic substrates are not specific for plasmin and non-plasmin esterase activity may produce false assay values. Schultz et al., (1957) obtained unsatisfactory results using lysine ethyl ester and tosylarginine methyl ester as substrates for the determination of the fibrinolytic activity of

normal human plasma and this was confirmed by von Kaulla and Schultz (1958).

Cole and Mertz (1961) demonstrated that the fibrinolytic activity of both bovine and human plasminogen preparations parallels their caseinolytic activity in a linear relationship but that there was no such relationship between the fibrinolytic and esterolytic activities.

#### Immunochemical Method.

An immunochemical method based on the Ouchterlony agar gel diffusion technique using as antibody a serum prepared from rabbits which had been injected with an extract of fraction III was used by Hagan, Ablondi and De Renzo (1960). It is possible that when the antigen has been isolated in a pure form this method may be valuable.

#### Assays of Inhibitors.

The difficulty in assaying various components of the fibrinolytic system is illustrated par excellence by the difficulty in assaying antiplasmin(s). This has been due to difficulty in preparing potent plasmin free from plasminogen and from activator and also due to the lack of agreement on an international plasmin unit. Plasminogen preparation and activation (Fishman and Kline, 1956; Kline and Fishman, 1961 a) is a time-consuming procedure. We have used a ready made active preparation 'Thrombolysin' with satisfactory results. Techniques have been used to determine antifibrinolytic properties based on the capacity of the plasma or serum under test to block the action of plasmin in a fibrinogen-thrombin clot indicator system. Bozzo, Piomelli and Schettini (1956) used a fixed amount of plasmin in varying dilutions of the serum under test, whereas, Biezenski (1960) added his plasmin in various dilutions to fixed amounts of the test serum. Since serum and plasma contain the same amount of antifibrinolysins (Grob, 1949;

Biezenski, 1960) either can be used. The second method has been successfully used in this study. Biezenski found the level of antifibrinolysins to be stable in normal individuals.

Methods based on anticaseinolytic properties estimate either (1) the difference between caseinolytic activity of plasmin before and after incubation with the inhibitory substance (Sherry, Lindemeyer, Fletcher and Alkjaersig, 1959), or (2) the differences in caseinolytic activity of activated and inactivated euglobulins (Berry, 1962), or (3) the difference in activity of whole serum and of euglobulin fraction (Quie and Wannamaker, 1960). All available methods of inhibitor estimation are at the best semi quantitative.

#### Rapid Fibrinogen Determinations (Hardisty, 1958)

Rapid fibrinogen assays may be required in cases of excessive fibrinolysis and/or in "hyperplasminaemia". The standard methods of plasma fibrinogen estimation in which a chemical determination of the protein content of the fibrin clot is used are too time-consuming to be of immediate practical value in such cases, and are inaccurate at fibrinogen levels of less than 35 mg. per 100 ml. These methods, which will not be considered here, may be used to confirm results obtained by more rapid procedures.

For rapid determination of fibrinogen for example in the acute defibrination syndrome the following tests are useful.

1. Whole blood clotting time.
2. Thrombin clotting time of plasma.
3. Turbidimetric fibrinogen assay.

(1). In the clotting time test, if a solid clot forms within ten minutes, there is no significant deficiency of fibrinogen. Fibrinogen levels below 100 mg. per 100 ml. result in the formation of small friable

clots and possibly in some prolongation of the clotting time. If the fibrinogen level falls below 30 mg. per 100 ml., no visible clot may be formed.

(2). Thrombin Time. A small friable or filamentous clot, usually associated with a prolonged clotting time, denotes fibrinogen deficiency. Complete failure of clot formation in plasma denotes a fibrinogen level below about 10 mg. per 100 ml.

(3). Turbidimetric Assay. Fibrinogen is precipitated from plasma using ammonium sulphate in a final concentration of 13 per cent. The fibrinogen concentration of the plasma is determined from photometric readings by reference to a standard calibration curve.

Recently a rapid immunological test (Fi test, Hyland Laboratories, California) has been highly recommended by Fletcher, Alkjaersig and Sherry (1962 a).



"B"

METHODOLOGY

TECHNIQUES USED IN THE INVESTIGATION  
OF THE FIBRINOLYTIC SYSTEM.

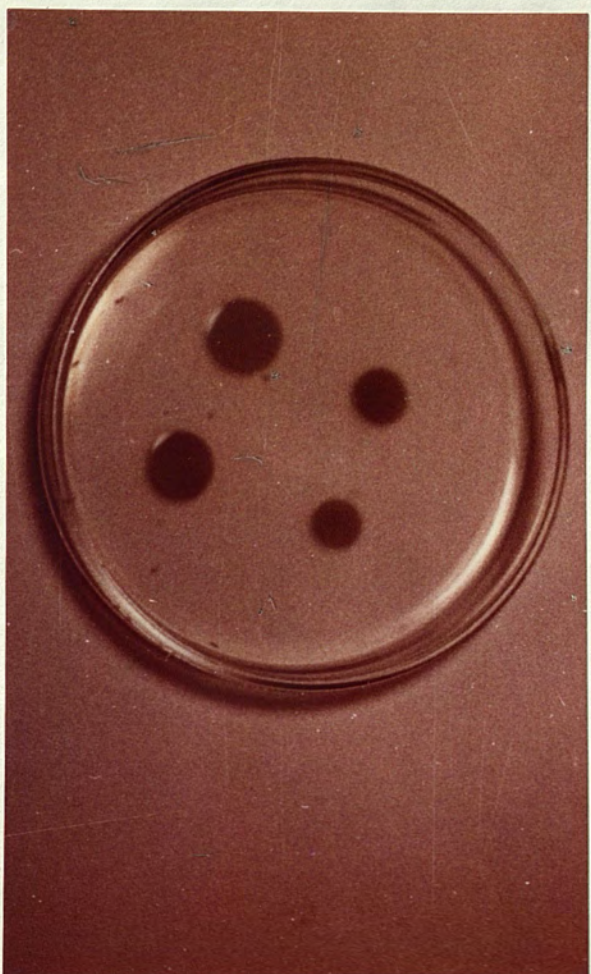


Figure 3      Heated fibrin plate.



The technical methods employed in these cases have previously been reported in the literature but have been modified slightly in most cases.

(1) Heated Fibrin Plate Technique.

Reagents.

1. 0.4% bovine fibrinogen (Armour) in veronal acetate buffered saline, pH adjusted to 7.4 if necessary with N/10 NaOH or N/10 HCl, and kept frozen at  $-20^{\circ}\text{C}$ .
2. Veronal acetate base: 9.714 G. sodium acetate trihydrate, 14.714 G. sodium diethyl barbiturate; made up to 500 ml. with distilled water (D.W.)
3. Veronal acetate buffered saline: 25 ml. veronal acetate base (see Reagent No. 2 above), 25 ml. N/10 hydrochloric acid, 450 ml. normal saline; pH adjusted to  $7.4 \pm 0.05$  (glass electrode).
4. Thrombin (Maw): 200 National Institutes of Health (N.I.H.) units per ml. in buffered saline.
5. Bromothymol blue: 0.04% in 95% ethyl alcohol.
6. Flat-bottomed Petri dishes 9 cm. diameter carefully selected for a uniform flat base; conical flasks about 30 ml. capacity and filter paper.

Method.

To 10 ml. of fibrinogen solution in a conical flask add 0.1 ml. thrombin and quickly mix the contents and then pour into a Petri dish avoiding contamination by air bubbles. After clotting has occurred incubate the plate at  $37^{\circ}\text{C}$ . for 10 minutes. Apply a damp filter paper to the inside of the topcover of the Petri dish and heat the plate at  $85^{\circ}\text{C}$ . in a hot air oven for 45 minutes. After cooling to room temperature,

inoculate the plate with a standard drop (0.03 ml.) of the test material. Duplicate this on the same plate, then incubate the plate overnight (about 18 hours) at 37°C. Keep the plates absolutely horizontal throughout. Outline the lytic areas by a drop or two of bromothymol blue (Soardi, Stefanini, Stump and Marin, 1960). See figure 3 of the fibrin plate. The application of local sodium hydroxide which the last authors recommend has proved unsatisfactory in this study by causing rapid non-specific digestion of the fibrin.

The results are expressed by calculating the areas of lysis which, however, are not related to the enzyme concentration in a simple arithmetical ratio but the logarithms of the areas of lysis are directly proportional to those of the plasmin concentrations. This is shown in figure 4 where the test has been carried out on serial dilutions of a solution of plasmin 'Thrombolysin', Batch N<sup>o</sup>. C-1423 in distilled water. A hundred per cent solution is one that contains 150 mg.% of the enzyme.

Tables II and III show the results using heated fibrin plates.

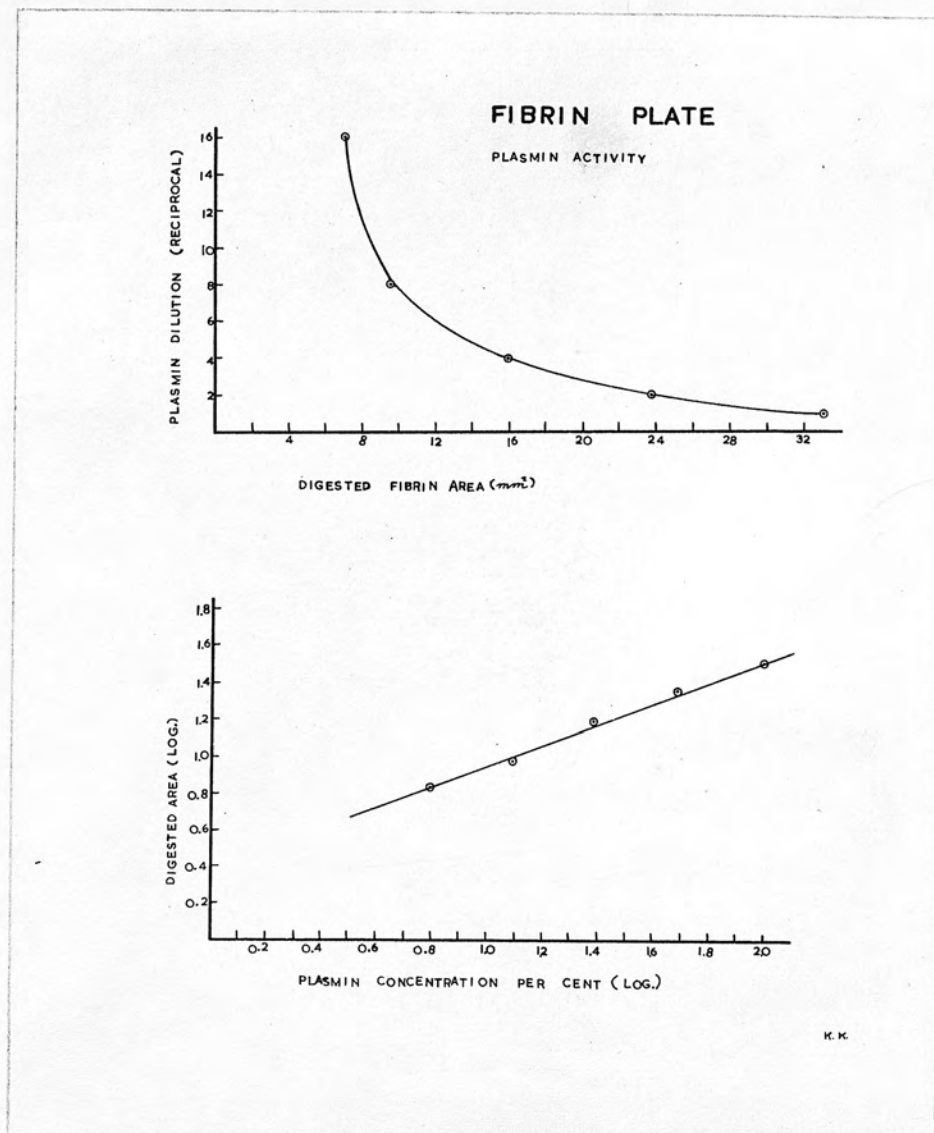
Table II

Lysed fibrin area in mm <sup>2</sup>	Plasmin dilutions of 150 mg% solution
33.19	1/0
23.76	1/2
15.91	1/4
9.62	1/8
7.07	1/16

Table III

Lysed fibrin area (log. of mm <sup>2</sup> )	Plasmin concentrations (log. of percentage)
1.52	2.00 (100 %)
1.37	1.69 ( 50 %)
1.20	1.39 ( 25 %)
0.98	1.09 (12.5%)
0.84	0.79 ( 6.25%)

Normal areas of lysis for the euglobulin fractions of plasma (see E.L.T.) are 20 to 50 mm<sup>2</sup> and measure plasma plasmin activity.



**Figure 4** Application of heated fibrin plate for the study of plasmin 'thrombolytic' activity. A hyperbolic curve is obtained by plotting the digested fibrin area in mm<sup>2</sup> against plasmin dilution expressed as reciprocal. The curve is changed to a straight line when the lysed area and plasmin concentration per cent are both expressed logarithmically.



(2). Plasma Euglobulin Lysis Time (E.L.T.)Reagents

1. Plasma: This is prepared from a mixture of nine parts of whole blood with one part of 3.8% sodium citrate (B.P) solution. Plasma processing may begin immediately or the specimen may be kept for few days at  $-20^{\circ}\text{C}$ . Standardization of the procedure is essential.
2. One per cent acetic acid.
3. Wash solution: 450 ml. D.W. + 25 ml. normal saline + 1% acetic acid to bring the pH to 5.2.
4. Veronal acetate buffered saline : (see fibrin plate method)
5. M/40 calcium chloride.
6. Scrupulously clean test tubes, pointed glass rods, pH indicator paper and distilled water.

Method

The test is done in duplicate. To 0.5 ml. plasma, 9 ml. of distilled water are added and the pH brought to 5.2 with 1% acetic acid. The precipitate is kept at  $4^{\circ}\text{C}$ . for 20 minutes, centrifuged at 3,000 r.p.m. for 5 minutes and washed while undisturbed, with wash solution. The inside of the tube is carefully wiped dry and the precipitate dissolved completely in 0.5 ml. veronal acetate buffered saline stirring with a glass rod. After two minutes in a  $37^{\circ}\text{C}$ . water bath, the dissolved euglobulin solution is clotted with 0.5 ml. M/40 calcium chloride. For heparinized specimens from patients submitted to open-heart surgery on the heart-lung machine, the precipitate is dissolved in one ml. buffered saline and clotted with 0.05 ml. thrombin solution (Thrombin, Topical, Parke, Davis & Co), 200 N.I.H. units/ml. of distilled water. The basic pattern of the euglobulin lysis is that of a slow onset followed by increasing disintegration

speed of the coagulum with paracoagulation (Derechin, 1955) occurring at intervals. Complete lysis of the clot(s), which may be quite unheralded is expressed in minutes and is the euglobulin lysis time. In our hands values from 120 to 330 minutes with rarely a prolongation of this upper limit have been found in normal adult individuals on morning specimens. On afternoon specimens, normal values as low as 80 minutes have been encountered. This can be expressed as a rate (Buckell, 1958) using the equation:

$$\text{Rate} = \frac{1000}{\text{lysis time in minutes}}$$

Maximum normal rates of 8 and 12 for morning and afternoon specimens respectively have been obtained for overall fibrinolytic and/or activator activity.

### (3) Caseinolytic Assay (Biggs & Macfarlane, 1962).

#### Reagents

1. Phosphate solution: 0.1 M disodium hydrogen orthophosphate in 0.9% NaCl, pH 7.6.
2. 5% casein\*in phosphate solution. Hammersten casein (Hagan, Ablondi and De Renzo, 1960) and Pfanstiehl casein (Bundy and Mehl, 1958) have been used with success by the respective authors.
3. N/6 NaOH
4. 10% trichloroacetic acid.
5. Streptokinase 2000 units/ml. phosphate solution (Varidase-Lederle).
6. N/6 HCl
7. Citrated plasma (see euglobulin lysis time p. 47)

#### Method.

To 0.5 ml. plasma is added 0.5 ml. N/6 HCl and the mixture kept at room temperature for 15 minutes, after which 0.5 ml. of N/6 NaOH is added

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\*(Locally purified casein according to the method of Remmert and Cohen (1949) has been made and supplied by the Blood Transfusion Service, Edinburgh).

followed by 1 ml. of 0.1 M phosphate solution, 0.5 ml. SK solution and 2 ml. of 5% casein. The mixture is incubated at 37°C. After 2 minutes 2.5 ml. is withdrawn from the incubated mixture and added to 2.5 ml. of 10% trichloroacetic acid (tube A). After 10 minutes of incubation of the precipitate in tube A at 37°C. (to ensure complete precipitation of undigested casein and possibly large polypeptide molecules) the contents are filtered through a Whatman No.1 filter paper. After 62 minutes of incubation of the original mixture, 2.5 ml. of 10% trichloroacetic acid is added to the remaining 2.5 ml. of the mixture and the precipitate filtered after a further 10 minutes incubation.

The difference in degree of caseinolytic breakdown in both solutions A & B is measured as a trichloroacetic acid - soluble tyrosine moiety in a Unicam SP 500 spectrophotometer at a wavelength of 280 millimicrons. The caseinolytic activity is arbitrarily expressed as the increase in optical density of the trichloroacetic acid filtrate after 60 minutes incubation, multiplied by 1000. Normal values are 239 - 443 units of plasminogen.

#### (4). Assay of Antifibrinolytic Activity.

##### Reagents.

1. Fibrinogen solution: Human fibrinogen as Cohn fraction I (supplied by Blood Transfusion Service, Edinburgh) is used in a concentration of 250 mg.% in veronal acetate buffered saline (see under fibrin plate) but is stored in a tenfold concentration at - 20°C. and diluted to the required concentration immediately before use.
2. Thrombin (Maw): 10 N.I.H. units per ml. distilled water.
3. Plasmin solution: "Thrombolysin" is used in a predetermined strength which just lyses the clot at minimal plasmin dilution in the indicator system and this concentration is determined afresh for each new batch of plasmin solution used. For the batch we used (C-1423)

this was found to be on an average 150 mg.% in distilled water. Plasmin solution is stored in 2.5 ml. amounts at  $-20^{\circ}\text{C}$ . and is stable for a few months.

4. Plasma: Citrated plasma (see E.L.T. p.47 ) is diluted  $1/20$  with buffered saline immediately before use.

#### Method.

A series of 12 dilutions ranging from  $1/2$  to  $1/24$  of the plasmin solution in use is made in buffered saline as the diluent. To each of a set of 12 tubes is added 0.1 ml. of the plasmin dilution and 0.1 ml. of the diluted test plasma. The tubes are then incubated at  $37^{\circ}\text{C}$ . for 12 minutes and 0.2 ml. of fibrinogen solution and 0.2 ml. of thrombin solution are then added in quick succession. When clot formation is complete the mixture is left undisturbed at  $37^{\circ}\text{C}$ . for exactly 30 minutes. Positive and negative controls are set up, the former of plasmin in buffered saline and these tubes should all show lysis and the latter of buffered saline in plasma and in these tubes there should be no lysis.

The result is expressed as the titre at which the lowest plasmin dilution fails to lyse the indicator clot. The results can also be expressed as a percentage of normal, calculated by comparing the titre obtained using the solution under test, with that of normal plasma assayed at the same time, or it can be expressed in arbitrary units whereby one unit indicates the activity of a  $1/24$  dilution and 12 units that of a  $1/2$  dilution of plasmin solution. Each unit is an increment in reciprocal of two. Normal plasma antifibrinolytic values are 4-8 units or  $1/8$  -  $1/10$  titres.

"C"

INVESTIGATIONS INTO THE FIBRINOLYTIC SYSTEM IN CERTAIN  
CONGENITAL AND HEREDITARY HAEMORRHAGIC DISORDERS :  
A CRITICAL EVALUATION OF THE THEORY OF DYNAMIC HAEMOSTASIS.





### Introduction

It is now accepted that excessive fibrinolytic activity can occur in a wide variety of physiological and pathological states and not uncommonly in the latter this can give rise to abnormal bleeding (Tagnon, Schulman, Whitmore and Kravitz, 1952). The effect of the fibrinolytic mechanism on coagulation is mediated through two main channels: (a) the digestion of certain coagulation factors, mainly factor I (fibrinogen), factor V and factor VIII (antihaemophilic globulin) (Koller, 1961) and (b), by defective fibrin polymerization (Alkjaersig, Fletcher and Sherry, 1962). In spite of such relation between fibrinolysis and some coagulation factors and of the possible role, subsequently, of an increased fibrinolytic activity in providing a trigger action for, or enhancing an existing coagulation defect in haemorrhagic states (see p. 4), reports on the fibrinolytic system in these cases have been infrequent.

Mole in 1948 was the first to draw attention to the possible physiological role of fibrinolysis in preventing thrombi from being formed on the vascular endothelium in health and suggested that atherosclerosis might result from the failure of this defence mechanism. Copley (1954; 1957) postulated that, in vivo, there existed a dynamic equilibrium between two opposing forces, the one depositing fibrin on the vascular endothelium (the coagulation mechanism) and the other constantly removing it (the fibrinolytic mechanism). A balanced harmony between the actions of these two processes helps to maintain the integrity of the vascular endothelium. According to this theory a disturbance of the balance might result in abnormal fibrin deposition, subsequently leading to atherosclerosis and/or thrombus formation, or produce a haemorrhagic state. This view has received support from other workers (Astrup, 1956; 1956 a; Jensen, 1956; Fearnley, 1961) for it has offered an attractive and apparently accepted

explanation for the perplexing pathogenesis of the thromboembolic syndromes. Further support has been given to it by the findings of a decreased fibrinolytic activity after myocardial infarction and intermittent claudication (Nestel, 1960) and after fatty meals (Tilliman, O'Neal, Thomas and Hixon, 1960).

The concept of a physiological dynamic equilibrium does, however, not accord with the findings of other workers of a lack of such correlation between fibrinolysis and atherosclerotic heart disease (Berry, 1962) nor between fibrinolysis and lipaemia (Hougie and Ayers, 1960; Blix, 1961). Lewis, Ferguson and Schoenfeld (1961) questioned the theory after they demonstrated that anticoagulants did not lengthen fibrinogen survival time in dogs. Doubt was also cast on the theory from the studies of fibrinogen turnover in haemophilia which was shown to compare with that in normal individuals (Rausen, Cruchaud, McMillan and Gitlin, 1961). Oiwini, Oiwini and Baluda (1962) similarly questioned the validity of the theory when they found that there was no change in the rate of passage of an Evans blue-albumin complex through the vascular wall during an induced fibrinolytic state in dogs.

If this theory holds true ~~then~~ a change in one of the components of the equilibrium state, for example a delayed or diminished coagulation mechanism as in certain haemorrhagic disorders, should lead to a balancing diminution in the other component and hence to a decrease in fibrinolytic activity. This postulate forms the basis of the present investigations in certain haemorrhagic disorders.

#### Materials and methods.

Thirty-seven successive patients suffering from various haemorrhagic disorders, all in a clinically quiescent stage, were selected for study from the regular attenders at the afternoon Out-Patient clinic for Coagulation

Disorders. Of these thirty-seven patients, twenty-four had factor VIII deficiency (classical haemophilia), four had factor IX deficiency (Christmas disease), seven had von Willebrand's disease and two had hereditary haemorrhagic telangiectasia. Six of these haemostasis deficient patients were studied while undergoing operative procedures (five for dental extractions and one for a minor orthopaedic operation). In all six, the operations were done under general anaesthesia and under transfusion cover of fresh frozen plasma and/or human antihaemophilic fraction prepared by Blombäck method. No abnormal bleeding developed during these operations. Blood samples were taken on the morning immediately prior to the operation, on completion of the operation and on the first day post-operatively.

Blood was taken by clean venepuncture (through a siliconed needle hypodermic, 21 SWG) into a siliconed syringe and mixed with 3.8% sodium citrate solution (B.P.) in the proportion of 9 parts of blood to 1 part of citrate solution.

The fibrinolysin and/or activator activity, the antifibrinolysins, the plasminogen and plasmin contents of the plasma were measured by above detailed techniques. The fibrinogen was estimated by the Kjeldahl method (Normal = 200 - 500 mg.%).

### Results.

The values for the parameters measured in the quiescent phase of the patients under investigation are shown in table IV. Table V gives the values obtained in six of these patients over the operation phase.

Table IV shows that the 'fibrinolytic activity' and/or the 'activator activity' as well as the fibrinogen values were within normal limits during the quiescent phases in the patients investigated and that the antifibrinolytic activity was within the normal range in twenty-five cases, moderately increased in six (Nos. 1, 9, 12, 14, 17 and 31) and moderately decreased in

the other six cases (Nos. 21, 22, 24, 28, 35 and 37).

Of twelve patients plasminogen assays were normal in ten, but of these ten, three were high normal (Nos. 7, 9 and 23), one of these three (No. 7) having in addition a high plasmin level, and one other of these ten (No. 37) having a low normal plasminogen level. Of the remaining two patients one showed a definite decrease in plasminogen together with an increase in plasmin (No. 17) and the other case (No. 33) showed an increase in plasminogen.

Out of eleven patients in whom fibrin plates were done, nine showed normal and two (Nos. 7 and 17) high, plasmin levels. All six cases undergoing operative procedures showed a transient increase in fibrinolytic activity which had reverted to normal on the first post-operative day. The antifibrinolysin(s) steadily increased in four cases (Nos. 1, 2, 3 and 4), diminished in one (No. 5) and remained constant in the last case (No. 6). Fibrinogen was ultimately raised in five cases and minimally changed in one case (No. 3). In three cases (Nos. 2, 3 and 5) a rise of fibrinogen was preceded by an initial drop in its level, concurrent with the increase in fibrinolytic activity.

#### Discussion.

In previous reports the fibrinogen level in haemophilic blood has more than once been reported as normal quantitatively as well as qualitatively (Sjölin, 1959). Results of our investigations in clinically quiescent phases of various bleeding disorders confirm this finding. Occasional results of plasminogen, plasmin and antiplasmin values outwith the normal range obtained in these studies may possibly be, in some cases at least, manifestations of subclinical activity of the haemorrhagic diathesis. Marked increase in antifibrinolytic activity has been found



sporadically even in normal people (Biezenski, 1960). These findings make speculation very hazardous as to whether an exaggerated fibrinolytic activity is the cause or the result of a spontaneous crisis in a bleeder. The response of the fibrinolytic mechanism to operative procedures found here conforms with what has been previously reported with operative procedures viz transient rise of fibrinolytic activity (Macfarlane, 1937; Sforza and Pozzi, 1960 a) with sometimes an opposing increase in antifibrinolysins (Sandberg, Tsitouris and Bellet, 1960). An ultimate rise of fibrinogen is expected as a result of the operative stimulus. It seems therefore that the fibrinolytic system is normal in these reported diseases in most of which there is known to be a delay in the haemostatic mechanism, and that its activation during and following operation followed a normal sequence.

It has been mentioned earlier that the theory of dynamic physiological equilibrium governing coagulation and fibrinolysis - a theory which has no substantial proof - has been criticised in different respects:(a), anticoagulants do not lengthen fibrinogen survival times (Lewis, Ferguson and Schoenfeld, 1961); (b), fibrinogen turnover in haemophilia is the same as that in normal individuals (Rausen, Cruchaud, McMillan and Gitlin, 1961) and (c), there is no change in the rate of passage in fibrinolytic states of an Evans blue-albumin complex through the vascular endothelial wall nor in its rate of excretion from the body (Oiwini, Oiwini and Baluda, 1962). Further, if deposited fibrin is normally under continuous removal one would expect to find an effective level of the active fibrinolytic enzyme (plasmin) in the circulation of normal individuals in order to carry out this function. This is known not to be the case and no free plasmin appears in circulating plasma (McNicol, 1962).

Evidence therefore suggests that this over simplification of the



coagulation-fibrinolytic mechanism is not true and that its role in the aetiology of atherosclerosis and of thromboembolic disease is not clear. It could be that the fibrinolytic mechanism enters into action in 'alarm' conditions; the role of physical and mental stress in enhancing the fibrinolytic activity is well established. This is substantiated by a recent report in which a technique was developed for teeth extractions in haemophiliacs that has eliminated the need for plasma or blood transfusion, and shortened considerably, or made unnecessary, any hospitalization (Lucas, Carroll, Finkleman and Tocantins, 1962). The method reported took advantage of hypnotic suggestion to avoid or alleviate all excessive physical and psychological stress before, during and after the operative procedure.

#### Summary

Investigation of the fibrinolytic system in a total of assorted thirty-seven cases of congenital and hereditary disorders of the haemostatic mechanism was carried out when these patients were clinically quiescent and when six of them underwent operative procedures.

On the basis of the parameters investigated, the fibrinolytic system was found to be normal in these individuals and to react normally to operative trauma.

It was concluded that if the theory of a physiological dynamic equilibrium between coagulation and fibrinolysis holds true, a delay in the initial process such as occurs in most of the patients investigated should be balanced by a decrease in the second process. Our results show no evidence that such a disturbance occurs and it was suggested that the fibrinolytic mechanism may enter into action in 'alarm' conditions.

Table IV

## Components of the Fibrinolytic System in Some Congenital and Hereditary

## Haemorrhagic Disorders

No.	Patient	Sex	Diagnosis	Euglobulin lysis time (min.)	(rate) (mm <sup>2</sup> .)	Plasmin	Plasminogen (unit)	Fibrinogen (mg.%)	Antifibrinolysin (titre)	(unit)
1	F.M.	M	Haemophilia	148	6.7			352	1/7	9.5
2	K.M.	M	"	130	7.6	44.19	417	408	1/10	8
3	G.M.	M	"	110	9			220	1/12	7
4	A.C.	M	"	117	8.5	35.79	348	239	1/16	5
5	I.P.	M	"	195	5.1			275	1/10	8
6	J.W.	M	"	180	5.5			266	1/18	4
7	B.M.	M	"	140	7.1	70.91	443	209	1/12	7
8	W.A.	M	"	240	4.5			275	1/16	5
9	R.M.	M	"	180	5.5	33.19	442	332	1/9	8.5
10	J.W.	M	"	135	7.4			365	1/17	4.5
11	D.M.	M	"	109	9.1			478	1/14	6
12	E.M.	M	"	112	8.9			344	1/8	9
13	M.C.	M	"	209	4.7			305	1/18	4
14	M.R.	M	"	150	6.6			330	1/6	10
15	J.M.	M	"	95	10.5			259	1/18	4
16	J.P.	M	"	122	8.1			310	1/18	4
17	R.M.	M	"	145	6.8	56.76	227	338	1/6	10
18	J.M.	M	"	93	10.7			313	1/12	7
19	T.M.	M	"	90	11.1			288	1/18	4
20	A.A.	M	"	197	5			382	1/16	5
21	J.B.	M	"	116	8.6			275	1/22	2
22	G.M.	M	"	110	9	47.19	297	242	1/20	3
23	S.P.	M	"	230	4.3	44.19	442	372	1/16	5
24	R.S.	M	"	150	6.6			349	1/22	2
25	I.D.	M	Christmas disease	140	7.1			253	1/18	4
26	J.S.	M	"	180	5.5			200	1/10	8
27	R.W.	M	"	145	6.8	38.50	299	309	1/16	5
28	J.D.	M	"	150	6.6			226	1/20	3

Table IV (contd.)

No.	Patient	Sex	Diagnosis	Euglobulin lysis time (min.)	(rate) (mm <sup>2</sup> .)	Plasmin (mm <sup>2</sup> .)	Plasminogen (unit)	Fibrinogen (mg.%)	Antifibrinolysis (titre)	(unit)
29	S.H.Jr.	M	von Willebrand's disease with factor VIII deficiency	120	8.3			231	1/11	7.5
30	S.H.Sr.	M	"	120	8.3			365	1/18	4
31	F.F.	F	"	105	9.5			319	1/2	12
32	A.G.	F	"	240	4.5			213	1/16	5
33	J.R.	M	von Willebrand's	110	9		479	230	1/10	8
34	A.S.	F	disease without	172	5.8			340	1/16	5
35	I.R.	F	factor VIII deficiency	209	4.7	33.19	247	339	1/24	1
36	J.J.	M	Hereditary haemorr-	126	7.9	38.50	266	256	1/12	7
37	A.D.	F	hagic telangiectasia	77	12.9	38.50	239	382	1/22	2

Table V

Changes in the Fibrinolytic System in some Congenital and Hereditary Haemorrhagic Disorders with Minor Operative Procedures. (ELT = euglobulin lysis time in minutes, FN = fibrinogen in mg.%, AF = antifibrinolysin(s) units).

No.	Name	Sex	Diagnosis	Operation	Test performed	Pre-operative	Immediately post-operative	First post-operative day
1	C.M.	M	Haemophilic	Orthopaedic operation	ELT FN AF	205 222 6	162 425 8.5	
2	B.M.	M	"	Dental extractions	ELT FN AF	235 365 7	60 321 7	250 424 8.5
3	J.W.	M	"	"	ELT FN AF	135 365 4.5	130 352 6.5	138 357 7
4	J.A.	M	"	"	ELT FN AF	218 201 2	116 375 5	202 340 8
5	J.D.	M	Christmas disease	"	ELT FN AF	179 226 3	101 211 1	201 354 1
6	A.G.	F	von Willebrand's disease with factor VIII deficiency	"	ELT FN AF	240 213 5	105 283 5	142 363 5

"D"

CHANGES IN THE FIBRINOLYTIC SYSTEM COMPONENTS DURING  
EXTRACORPOREAL CIRCULATION



### Introduction

In 1954, Gibbon after many years of experimental work achieved the first successful total body perfusion in a human body using a form of cardiopulmonary bypass. A prime aim of all workers in this field since has been to produce safe and adequate anticoagulation of the blood during such a procedure and to reverse it swiftly and safely at the end of the operation. Furthermore it was necessary that there should be none, or at the most, a minimal and insignificant alteration in both the cellular and humoral elements of the blood due to this procedure and although satisfactory results have been achieved we are still far from perfection. Reports of haematological changes during extracorporeal circulation have varied particularly in the coagulation mechanism.

There has been no general agreement on the occurrence of bleeding tendencies, and, where these have been reported, there has been a wide diversity of opinion as to their cause. Many theories have been advanced to explain the aetiology of excessive bleeding during extracorporeal circulation in open-heart surgery and among these have been pyrogens, type of apparatus, duration and rate of perfusion (Brown and Smith, 1958), inadequately cleaned and sterilized equipment, incompatible blood or inadequate haemostasis (DeWall, Long, Gemmill and Lillehei, 1959), active trauma (Osborn, MacKenzie, Shaw, Perkins, Hurt and Gerbode, 1955), massive blood transfusion (Matzke, Jensen and Rygg, 1961), heparin rebound phenomena (Holemans, Vermeylen and Verstraete, 1960) de novo development of circulating anticoagulants (von Kaulla and Swan, 1958), activation of the thromboplastin mechanism with consumption of essential coagulation factors (Ollendorff, Storm,

Rygg and Arnfred, 1961), secondary hypocoagulation after heparin neutralization (Matzke, Jensen and Rygg, 1961), type of cardiac lesion (Gans, Lillehei and Krivit, 1961) and other unknown causes (Cooley, Belmonte, DeBakey and Latson, 1957).

Thrombocytopenia, constantly follows the procedure and it is believed that it can cause a serious bleeding disorder (Perkins, Osborn, Hurt and Gerbode, 1956). Excessive fibrinolysis, however, has been the next most commonly reported disturbance and has sometimes given rise to serious bleeding (Perkins, Osborn and Gerbode, 1958). Activation of the fibrinolytic system has since been shown to be conditioned by an in vivo factor, and this has sometimes apparently been dependent on the rate and duration of perfusion and on the pH changes occurring (von Kaulla and Swan, 1958). The time of onset of maximum fibrinolytic activity during extracorporeal circulation has born no relation to any specific cause. Reports on alterations of components of the fibrinolytic system due to bypass, namely the fibrinolysins and fibrinogen have varied and little heed has been paid to the antifibrinolytic and the other inhibitor systems.

It is the object of this study to determine and discuss changes in the fibrinolytic system observed in eighteen patients with cardiac disease who were submitted to open-heart surgery and cardiopulmonary bypass.

#### Materials and Methods

Eighteen patients undergoing cardiopulmonary bypass and open-heart surgery for various cardiac diseases in the Royal Infirmary of Edinburgh formed the subject of this study. Table VI lists the relevant particulars of each patient. The patients were selected only on their

anticipated availability for obtaining adequate blood specimens at fixed times throughout their operative history.

Extracorporeal circulation using a Melrose N.E.P. rotating disc pump oxygenator was used with hypothermia down to a temperature averaging 30-31°C. The machine was primed using fresh heparinized blood (30 mg. heparin in 15 ml. sterile normal saline to which was added 400 ml. of fresh blood) collected on the morning of the operation and stored at 4°C. until needed. Blood donors had previously been screened routinely for abnormal antibodies, crossmatched with the patient and intermatched with one another.

The patients were heparinized immediately prior to cardiac cannulation using 3 mg. of heparin per kilogram of body weight and reversing it post-operatively with hexadimethrine bromide well diluted in sterile 5% dextrose given slowly in not less than ten minutes to avoid circulatory disturbance (MacKenzie, Wade, Davies and Zellos 1961). The neutralization of heparin was deemed complete when the thrombin time returned to pre-heparinization level and was not further shortened by the prior addition of toluidine blue to the test plasma. Additional doses of hexadimethrine bromide were given if required until this was achieved. Heparinization during bypass was considered adequate when the thrombin time was not less than 60 seconds (control 10-12") and the clotting time (Lee and White) not less than 60 minutes. During anticoagulation 10 ml. blood specimens were obtained as required from the circuit but at other times blood was drawn into heparin in a concentration of 5 units/ml. of blood. Specimens were taken (1) before anaesthesia, (2) a few minutes before bypass, (3) a few minutes after commencing bypass, (4) at the end of bypass, (5) a few minutes after reversal of the anticoagulation and (6) 30 minutes later.

Fibrinolytic activity and/or activator activity was measured by the euglobulin lysis time, modified for heparinized blood vide supra (p.47 ).

Antifibrinolysins and caseinolytic plasminogen assays were done according to above described methods (p. 48,49) Fibrinogen was estimated by the Kjeldahl technique, normal values being 200-500 mg.%

### Results

Table VII shows the results obtained in these tests.

Fibrinolysis: Excessive fibrinolysis was found in all of the cases at some time during the procedure. On 9 of these occasions the maximum fibrinolytic activity was found during bypass, on 7 occasions after hexadimethrine bromide reversal of the anticoagulation and on 2 occasions during anaesthesia but immediately prior to the onset of bypass. Enhanced fibrinolytic activity was transient and had abated 30 minutes at the most, after reversal of the coagulation mechanism. In spite of the demonstration of an abnormal fibrinolytic activity in all the cases, only one transient clinically significant fibrinolysis was met with (case No. 19) causing an uncontrolled haemorrhage for less than thirty minutes and it abated spontaneously. Apart from case No. 5 who died from cardiac arrest, all other cases recovered from the operative procedure and had no serious clinical disturbances of the haemostatic mechanism.

Fibrinogen: In all the cases and usually concomitant with a rise in fibrinolytic activity, a variable decrease in fibrinogen level was noted.

Antifibrinolysins: In 8 cases (Nos. 1,8,9,11,12,13,16 and 17) antifibrinolysins decreased during the operation, returned to normal and



sometimes even showed a slight increase above normal after reversal of anticoagulation post-operatively. A steady increase was found in 4 cases (Nos. 2,7,10 and 18) and a steady decrease in another 4 (Nos. 5,6,14 and 15). One case (No. 3) showed an increase in antifibrinolysins before returning to normal levels and there was virtually no change in one case (No.4).

Plasminogen: Of 7 cases in which plasminogen levels were determined 5 showed a decrease which had reverted spontaneously to pre-operative levels by the time the final sampling was done 30 minutes after the administration of the hexadimethrine bromide though one case showed a final rise in plasminogen (No. 14) and the other a final decrease in it (No. 13).

### Discussion

From this study of the fibrinolytic system during extracorporeal circulation in open-heart surgery, it would seem that excessive fibrinolysis is inevitable at some time(s) during the procedure, causing, however, no serious consequences when those described methods are used. It is suggested that mechanical trauma might play a part in this increased fibrinolysis, either per se, or through activating thromboplastin, since in most cases the maximum rise in activity has coincided with the end of the bypass. The occurrence of the same phenomenon in 7 cases following heparin neutralization might be explained by the removal of an inhibitory effect exerted by heparin on the fibrinolytic system though the role of heparin in fibrinolysis is not clear (von Kaulla and McDonald, 1958; Douglas, 1962).

The role of the antifibrinolysins is less certain and has been much less investigated. After some operations an increase of inhibitors

has been reported (Sandberg, Tsitouris and Bellet, 1960) whilst in some experimentally induced fibrinolytic states there has been an increase, followed by a decrease, in the antifibrinolysin titre (von Kaulla, 1958). The role of fibrinolytic inhibitors is at present not really known partly because of the complicated technical procedures employed in cardiopulmonary bypass and to the many drugs used during it.

Decreases in plasminogen and fibrinogen levels during cardiopulmonary bypass found in the present study accords with reports on similar decreases associated with a fibrinolytic state (Sawyer, et al., 1960; Nilsson and Olow, 1962).

#### Summary

The changes in the fibrinolytic system observed in eighteen patients with cardiac disease undergoing open-heart surgery on cardiopulmonary bypass with hypothermia was studied.

This investigation revealed that excessive fibrinolysis is inevitable at some time during the procedure, but need not, however, cause any serious consequences as shown in the patients studied. Plasminogen and fibrinogen diminution was noted, while changes in the antifibrinolysins were less clear.

Table VI

Clinical and perfusion data. (1) & (2) = On operation day and on first post-operative day respectively.

No.	Patient	Age (years)	Sex	Weight (kg.)	Diagnosis	Duration of bypass (minutes)	Rate of perfusion (ml./kg./min.)	Blood loss (ml./kg.)	Serious bleeding	Fate
1	G.H. 29/8/61	31	M	65	Aortic stenosis	70	49	(1) 2 (2) 3	None	Recovery
2	C.M. 6/10/61	4	F	17	Ventricular septal defect	40	95	(1) 40 (2) Negli- gible	None	Recovery
3	J.S. 10/10/61	54	M	54	Double mitral	85	59	(1) 5 (2) 5	None	Recovery
4	M.F. 7/11/61	57	F	55.75	Calcified aortic cusps	84	66	(1) 3 (2) 4	None	Recovery
5	E.S. 25/4/62	22	F	50	Mitral incompetence	88	64			Death from cardiac arrest
6	B.H. 30/5/62	9	F	26.25	Ventricular septal defect	31	87	(1) 25 (2) Negli- gible	None	Recovery
7	E.M. 6/6/62	24	F	47.50	Ventricular septal defect	83	76	(1) 20 (2) 23	None	Recovery
8	M.D. 12/6/62	8	M	22.5	Ventricular septal defect	47	93	(1) 40 (2) Negli- gible	None	Recovery
9	C.H. 20/6/62	15	F	54	Ventricular septal defect	34	69	(1) 44 (2) Negli- gible	For 30 minutes after reversal	Recovery

Table VI (contd.)

No.	Patient	Age (years)	Sex	Weight (kg.)	Diagnosis	Duration of bypass (minutes)	Rate of perfusion (ml./kg./min.)	Blood loss (ml./kg.)	Serious bleeding	Fate
10	L.N. 4/7/62	20	F	52	Ventricular and atrial septal defect	47	67	(1) 19 (2) Negli- gible	None	Recovery
11	E.N. 25/7/62	13	M	33.50	Ventricular septal defect	25	83	(1) 36 (2) 34	None	Recovery
12	D.O. 1/8/62	33	M	58	Ventricular septal defect	48	58	(1) 4 (2) 2	None	Recovery
13	K.L. 8/8/62	9	M	28.50	Ventricular septal defect	85	85	(1) 25 (2) 3	None	Recovery
14	V.R. 5/9/62	18	F	52	Atrial septal defect & cleft mitral valve	72	65	(1) 12 (2) 5	None	Recovery
15	J.T. 12/9/62	12	M	30.5	Ventricular septal defect & pulmonary stenosis	90	79	(1) 33 (2) 6	None	Recovery
16	L.C. 11/10/62	32	M	71	Aortic stenosis	52	48	(1) 8 (2) 4	None	Recovery
17	R.H. 25/10/62	37	M	77	Aortic stenosis	70	44	(1) 6 (2) 2	None	Recovery
18	J.T. 30/10/62	15	M	37.50	Ventricular septal defect	91	82	(1) 10 (2) 7	None	Recovery



Table VII

ELT = Euglobulin lysis time in minutes. AF = Antifibrinolytic activity units. FGN = Fibrinogen in mg. per cent. PGN = Plasminogen units.

No.	Patient	Test	Before anaesthesia	Before bypass	On bypass		After reversal of coagulation	
					After few min.	At the end	After few min.	After 30 min.
1	G.H.	ELT AF FGN	220 7 202	109 < 1 220	214		185	203 7.5 264
2	C.M.	ELT AF FGN		135 6 222	60		140 8 175	
3	J.S.	ELT AF FGN	> 300 7.5 282	100 9 Clot lysed before proper retraction	> 300 10 212	60 8.5 Clot lysed before proper retraction.	> 300 7 212	> 300 7.5 245
4	M.F.	ELT AF FGN	> 330 7.5 232	170 7 195	163 7.5 270		> 330 7 217	
5	E.S.	ELT AF FGN		> 300 8 400	200 6 375		70 4 clot lysed before proper retraction	
6	B.H.	ELT AF FGN	302 6 230	> 580 5 273	295 5 261		280 4 262	

Table VII (contd.)

No.	Patient	Test	Before anaesthesia	Before bypass	On bypass		After reversal of coagulation	
					After few min.	At the end	After few min.	After 30 min.
7	E.M.	ELT AF FGN	> 420 9 275	223 9 267	107 9 222		334 8 242	
8	M.D.	ELT AF FGN	> 506 3 243	> 488 2 235	305 4 288		431 3 180	430 6 246
9	C.H.	ELT AF FGN	> 360 11 307	> 360 10 300	216 9 181		11 7	333 8, one hour later 12 225
10.	L.N.	ELT AF FGN	> 300 1 225	> 300 1 238			Clot lysed before pro- per retraction 191	239, one hour later 300.
11	E.N.	ELT AF FGN FGN	> 360 10 231 323	117 10 173	> 360 9 183 252	138 9 199 354	179 4 156 33 10 141 344	> 300 5 269 360 10 137
12	D.O.	ELT AF FGN FGN	> 400 6 281 330	350 5 259 313	255 4 235 320	190 4 234 283	195 6 258 339	> 400 8 184 323

Table VII (contd.)

No.	Patient	Test	Before anaesthesia	Before bypass		On bypass		After reversal of coagulation	
				Before bypass	After few min.	After few min.	At the end	After few min.	After 30 min.
13.	K.L.	ELT AF FGN PGN	> 360 9 289 325	> 360 2 228 310	> 360 2 182 266	> 360 81 1 149 265	228 1 213 254	> 360 8 146 235	
14	V.R.	ELT AF FGN PGN	> 300 10 250 183	240 9 205 172	> 300 8 152 300	> 300 8 185 238	> 300 8 260 249	> 300 8 270 280	
15	J.T.	ELT AF FGN PGN	270 8 195 275		210 7 176 154	145 6 236 205	95 5 137 210	330 5 199 247	
16	L.C.	ELT AF FGN PGN	248 5 189 269	> 360 5 337 253	231 2 254 262	25 3 237 215	50 4 216 272	> 360 5 214 270	
17	R.H.	ELT AF FGN	> 300 1 225	> 300 220	> 300 1 190	105 < 1 170	26 1 200	> 300 3 231	
18	J.T.	ELT AF FGN	322 1 240	342 1 230	492 4 200	232 6 150	> 500 6 300	> 500 3	

PART TWO

A COMPARISON OF HAPTOGLOBIN PHENOTYPES IN HAEMOPHILICS  
AND NORMAL PERSONS IN SCOTLAND.



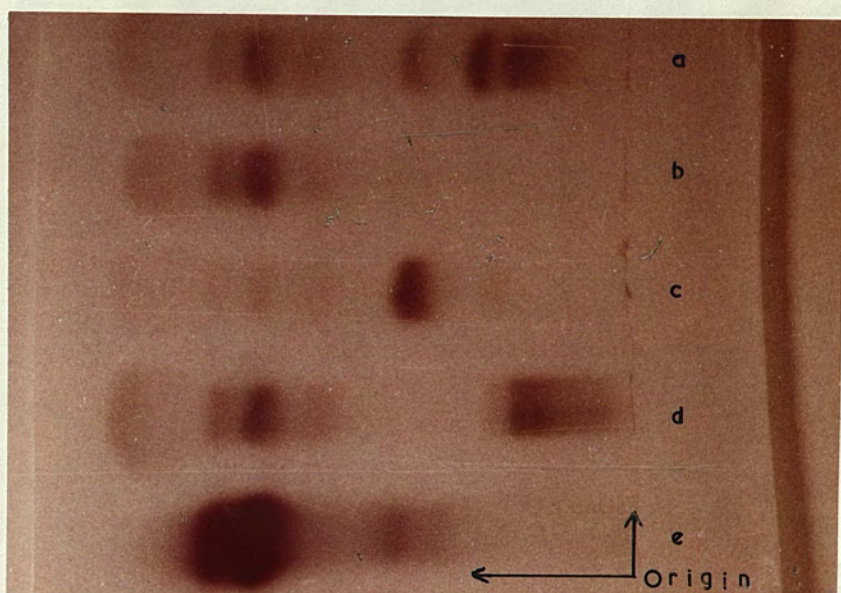


Fig. 5: a. Haptoglobin type 2-1, b. type 0-0, c. type 1-1,  
d. type 2-2, e. adult haemoglobin.

### Introduction

When normal plasma is subjected to electrophoretic analysis in free solution or in a supporting medium such as filter paper or cellulose acetate, six components can usually be identified; these are albumin, the alpha 1, alpha 2, beta and gamma globulins and fibrinogen. Quantitative variations in such components have been related to various disease states. When, however, normal plasma or serum was run electrophoretically on starch-gel by Smithies in 1955 he obtained nine to twelve fractions and from these he described three distinct genetically determined types of plasma protein patterns. The differentiation into these three types was based on variations in a protein fraction known as the 'haptoglobins'. Haptoglobins are glycoproteins which have the specific function of binding free haemoglobin liberated intravascularly and facilitating its elimination via the reticuloendothelial system (Eastham, 1961). Haptoglobins form part of the alpha 2 globulin fraction in conventional electrophoresis (Harris, Robson and Siniscalco, 1959); the haptoglobin-haemoglobin complex migrates with the beta 1 fraction, any free haemoglobin moving between the beta and gamma portions (Miale, 1962). The three main haptoglobin phenotypes are 1-1, 2-1, and 2-2. These phenotypes are determined by a pair of autosomal allelic genes,  $Hp^1$  and  $Hp^2$  (Smithies and Walker, 1955; Galatius - Jensen, 1957). Other inherited variants of the three common types of haptoglobins have recently been reported (Smithies and Connell, 1959).

Both ahaptoglobinaemia which may be either familial or arise spontaneously by mutation or by suppression of the  $Hp^1$  or  $Hp^2$  genes, and decreased concentration of serum haptoglobins may be found in disease states such as haemolytic diseases (Nosslin and Nyman, 1958). Racial



differences in haptoglobin types have been reported and ascribed to genetic factors such as G.-6-P.D. deficiency and to environmental factors such as malaria and liver disease (Mehta and Jensen, 1960). Quantitative changes in haptoglobins have been described in a variety of disease conditions in which there is inflammation or destruction of tissues as in acute pyogenic infection, rheumatic fever and malignancy (Jayle and Boussier, 1955), and in hepatobiliary disease (Owen, Mackay and Got, 1959). Phenotypic changes in the concentration of haptoglobins might, it is thought, be of some value in determining their physiological and genetic significance (Allison, 1958). No clear cut relationship has yet been found between haptoglobin types, transferrin types and specific clinical disorders; perhaps, however, as Allison (1958) points out, due to the relatively small number of individuals so far tested it would only have shown up had the correlation been high. Reports on such associations have been controversial (Sutton, Neel, Livingstone, Binson, Kunstadter and Trombley, 1959; Mehta and Jensen, 1960; Bennett, 1961; Bennett, Auricht, Gray, Kirk and Lai, 1961) and it has been recommended that work on this matter should be extended (Harris, Robson and Siniscalco, 1959).

It was considered desirable, therefore, to determine the haptoglobin phenotypes in patients with factor VIII deficiency and other hereditary haemostatic disorders in an endeavour to find yet another genetic link or tracer for them. There is already an infrequently reported occurrence of factor VIII deficiency with other genetically determined deficiencies of factors V, VII, IX and XI, with vascular and platelet abnormalities, and

with a freezing/serum factor (see p. 3 ). Of prime importance in this respect is the reported use of the genetic relation between factor VIII deficiency, red-green colour blindness, one form of muscular dystrophy, Xg blood groups and G.-6-P.D. deficiency in the study of the X chromosome cartography (Race, 1960; Tarleton, Race and Sanger, 1962). Of added interest is the discovery that factor VIII, a deficiency of which constitutes the basic defect in classical haemophilia, migrates in the alpha 2 and alpha 1 serum fractions in continuous flow electrophoresis (Lewis and Merchant, 1959) and it is known that the haptoglobins also migrate in the alpha 2 component (Harris, Robson and Siniscalco, 1959).

The pattern of distribution of several haptoglobin types within those human populations so far studied (Harris, Robson and Siniscalco, 1959; Arends and Rodríguez, 1960; Barnicot, 1961) may be part of a true polymorphism like that of blood groups affecting all human populations or be characteristic of certain areas of the world as are the haemoglobin variants. It has been thought likely that such polymorphisms may be maintained by a balance of selecting forces acting upon the different genotypes (Allison, 1958). The idea that people with certain blood groups are more susceptible to various diseases (Roberts, 1957) has not been universally accepted (Awny and Kamel, 1959; Wiener, 1962). It has been reported that the distribution of blood groups in haemophiliacs follows the normal pattern (Ikkala, 1960). The present study whose purpose is to investigate the genetically determined serum haptoglobin types in certain hereditary haemorrhagic disease conditions, might it seemed, help to elucidate these problems.

#### Material

Blood specimens were collected from 24 patients with factor VIII



deficiency (classical haemophilia), 5 with factor IX deficiency (Christmas disease), 7 with von Willebrand's disease and 2 with hereditary haemorrhagic telangiectasia all attending the Out-Patients clinic for Coagulation Disorders, The Royal Infirmary, Edinburgh. For normal local controls, blood was obtained from 100 consecutive pregnant healthy females of Scottish extraction referred to the Blood Transfusion Service, The Royal Infirmary, Edinburgh, for routine antenatal screening. These controls were considered to be satisfactory since the incidence of haptoglobin types does not vary with sex (Smithies, 1955) and, being congenitally determined, does not vary with the physiological process of pregnancy.

#### Methods

Haptoglobin typing has been done by starch-gel electrophoresis in a discontinuous system according to methods previously described (Kamel, 1959/1961).

The reagents used in this technique are:

1. Hydrolysed potato starch. Connaught Medical Research Laboratories, Toronto, Canada, batch No. 152. This was used in a concentration of 11.4 G. per 100 ml. gel mixing buffer.
2. Gel mixing buffer. This citrate buffer (0.076 M Tris, 0.005 M citric acid, pH 8.8).
 

Tris - (Hydroxymethyl) aminomethane	9.19 G./L.
Citric acid	1.00 G./L.
3. Bridge solution buffer (0.3 M boric acid, 0.05 M sodium hydroxide pH 8.4)
 

Boric acid	18.5 G.
------------	---------

Sodium hydroxide (N/1)	50 ml.
Distilled water up to	1000 ml.

#### 4. Stains

a. Benzidine stain. A mixture of 100 ml. distilled water, 0.5 ml. glacial acetic acid, 0.2 G. benzidine is warmed to dissolve the benzidine then 0.2 ml. of a 30% W./V. solution of hydrogen peroxide '100 vol.' are added. The benzidine stain should be prepared immediately before use.

b. Naphthalene black stain. Saturated solution.

Naphthalene black B 10	2.0 G.
Methanol-acetic acid solution	200 ml.

c. Methanol-acetic acid solution

Methanol	50 vol.
Glacial acetic acid	10 vol.
Distilled water	50 vol.

After saturating the haptoglobins with added haemoglobin the plasma or serum was subjected to electrophoresis in a starch-gel 25 x 15 x 0.6 cm. in dimensions. A direct potential of 120 volts, corresponding to a current of 9-11 milliamperes was applied for approximately 20 hours (overnight).

After completion of the run and slicing the gel through its thickness into two, one slab was treated with benzidine which clearly delineated the different haptoglobin types and the second slab was stained with naphthalene black for further confirmation and study.

Figure 5 illustrates the four types of haptoglobins shown as haptoglobin-haemoglobin complexes that have been found in this study. Type O-O individuals are those who show no haemoglobin-binding protein, but show

free haemoglobin in the starch-gel.

Because of the belief that there are two controlling allelic genes for each haptoglobin type and in order to compare the results statistically with previously reported studies (Mehta and Jensen, 1960; Bennett, 1961), individuals with ahaptoglobinaemia (type 0-0) were excluded in calculating the gene frequencies. Expected values for various haptoglobin phenotypes have therefore been calculated on the basis of the Hardy-Weinberg equation (type 1-1 =  $p^2$ , type 2-1 =  $2pq$  and type 2-2 =  $q^2$  where  $p = \text{Hp}^1$  gene frequency and  $q = \text{Hp}^2$  gene frequency).

### Results

Table VIII shows the haptoglobin types and estimated gene frequencies found in haemophilia, in some other haemostatic disorders and in one hundred local normal controls in this study. There were four phenotypic groups in the haemophilics and the controls: 1-1, 2-1, 2-2 and 0-0. Estimated gene frequencies cannot be deduced from the haptoglobin types in the cases of factor IX deficiency, von Willebrand's disease and hereditary haemorrhagic telangiectasia reported in this study because of the small numbers of patients involved.

### Discussion

Ahaptoglobinaemia which is a physiological state in newborn infants (Miale, 1962) was also found in 3 per cent of our normal adult controls, an incidence similar to that in England, 2.7 per cent (Allison, Blumberg and ap Rees, 1958) and in neighbouring European populations (Harris, Robson and Siniscalco, 1959; Arends and Rodríguez, 1960; Barnicot, 1961; Miale, 1962). The distribution of haptoglobin types in various populations is shown in table IX for purpose of comparison. The distribution of the phenotypes

1-1, 2-1 and 2-2 and the estimated gene frequencies  $Hp^1$  and  $Hp^2$  match those found in England and both are in accord with the average known gene frequency of  $Hp^1$  among Caucasoids, 0.34 - 0.46 (Arends and Rodríguez, 1960). Although there were apparently more  $Hp$  types 1-1 in the haemophilics (25%) than in the controls (10%) the difference is not statistically significant. An  $\chi^2$  test for the distribution of the three haptoglobin types in these haemophilics and in the local controls gives a value of 2.01 for two degrees of freedom. Accordingly  $p$  is more than 0.30 and this indicates that a real difference in the haptoglobin types of the two groups is very improbable (Smithies, 1955).

In our study, starch-gel electrophoresis of haemophilic blood has not shown any abnormal or characteristic zones which can be related to the  $\alpha_x$  globulin previously described (Bernfeld, Stefanini, Berkowitz and Hennessey, 1953). Factor VIII which is known to be present in plasma in very small amounts (Wagner, Brinkhous and Penick, 1959) has never been isolated in a pure form. It appears that the defect in factor VIII activity in classical haemophilia is not of a nature which would allow of its detection by the electrophoretic methods described. Although the number of patients with diseases other than haemophilia reported in this study are too small for statistical analysis of their haptoglobin types, their electrophoretic patterns showed no obvious abnormalities. More patients with these conditions will have to be investigated before any firm conclusions can be drawn.

In many diseases the distribution of haptoglobin types and/or blood groups has been found to accord with those in normal individuals (Craig and Wang, 1955; Allison and Blumberg, 1958; Awny and Kamel, 1959;



Bennett, 1961; Bennett, Auricht, Gray, Kirk and Lai, 1961; Gunson and Smithies, 1962), and Ikkala (1960) confirmed the normality of blood group distribution in haemophilics. In the present investigation no statistically significant difference was found between the haptoglobin phenotypes of haemophilics and of normal individuals.

#### Summary

Haptoglobin phenotyping by starch-gel electrophoresis was determined on 100 normal individuals in Scotland and on 38 patients with hereditary haemorrhagic diseases.

Haptoglobin type distribution in the controls accords with that of neighbouring populations.

No correlation between haptoglobin types and haemophilia could be detected.

TABLE VIII

Haptoglobin Phenotypes and Gene Frequencies in  
Scotland and in Certain Hereditary Haemorrhagic Disease States

Population	Values	Total	Haptoglobin types				Estimated gene frequencies	
			1-1	2-1	2-2	0-0	Hp <sup>1</sup>	Hp <sup>2</sup>
Normals	Observed	100	10	49	38	3	0.36	0.64
	Expected		12.57	44.70	39.73			
Factor VIII deficiency (classical haemophilia)	Observed	24	6	9	8	1	0.46	0.54
	Expected		4.87	11.42	6.71			
	Per cent		25.00	37.50	33.33	4.17		
Factor IX deficiency		5	2	3				
von Willebrand's disease		7	2	4	1			
Hereditary haemorrhagic telangiectasia		2			2			

Table IX

## Haptoglobin types in Various Populations

Population	Total	Haptoglobin types			Estimated gene frequencies		Reference
		1-1	2-1	2-2	0-0	$\frac{Hp^1}{Hp^2}$	
England	Obs. Exp. 179	33 33.1	88 87.7	58 58.2		0.43	Harris, Robson and Siniscalco (1959)
England (Oxford)	218 Per cent	10.1	55.5	31.7	2.7	0.38	Allison, Blumberg and ap Rees (1958)
England (London)	114 Per cent	17.6	48.2	34.2	0.0	0.42	Harris, Robson and Siniscalco (1959 a)
Australia (Caucasoids in Queensland)	100	14.0	58.0	28.0	0.0	0.43	Budtz-Olsen (1958)
Canada (Toronto)	49 Per cent	21.1	50.5	28.4	0.0	0.46	Smithies (1955)
U.S.A. (Caucasoids in Michigan)	68 Per cent	13.2	58.8	28.0	0.0	0.43	Sutton, Neel, Livingstone, Binson, Kunstader and Trombley (1959)
Denmark (Copenhagen)	2,046 Per cent	16.0	47.3	36.7	0.0	0.40	Galatius - Jensen (1958)
Norway (Oslo)	1,000 Per cent	13.2	46.2	40.6	0.0	0.36	Fleischer and Lundevall, (1958).
Spain (Basque)	107 Per cent	14.0	45.8	39.3	0.9	0.37	Allison, Blumberg and ap Rees (1958)

Table IX (contd.)

Population	Total	Haptoglobin types			Estimated gene frequencies		Reference
		1-1	2-1	2-2	Hp <sup>1</sup>	Hp <sup>2</sup>	
Italy (Cologna)	208	23 25.5	99 94.6	86 87.9	0.35	0.65	Harris, Robson and Siniscalco (1959)
Persia	34	2 2.1	13 12.8	19 19.1	0.25	0.75	Harris, Robson and Siniscalco (1959)
<u>Negroids</u>							
(1) Liberia & Ivory Coast	614	53.3	37.8	8.9	0.0	0.72	Sutton et al. (1959)
(2) Nigeria	99	53.5	11.1	3.0	32.3	0.59	Allison, Blumberg and ap Rees (1958)
Japanese (U.S.A.)	23	2 2.0	10 9.7	11 11.3	0.30	0.70	Harris, Robson and Siniscalco (1959).
Australia (Aborigines)							
a. Central Australia	100	40.0	47.0	13.0	0.0	0.63	Budtz - Olsen (1958)
b. North Queensland	123	12.0	68.0	20.0	0.0	0.46	Budtz - Olsen (1958)



PART THREE

PLASMA THROMBOPLASTIN GENERATION BLOCKING

ANTICOAGULANT IN HAEMOPHILIA

CLINICO-PATHOLOGICAL STUDY OF A REFRACTORY CASE

### Introduction

Since first demonstrated by Weil in 1906, circulating anticoagulants have been reported in cases of classical haemophilia, that is, haemophilia due to a deficiency of antihæmophilic factor, and closely allied blood coagulation disorders by many workers (Munro, 1946; Craddock and Lawrence, 1947; Singer, Mond, Hyman and Levy, 1950; Lewis, Ferguson and Arends, 1956; Verstraete and Vandenbroucke, 1956; Harmon, Zipursky and Lahey, 1957; Biggs and Bidwell, 1959; Sise, Gauthier, Desforbes and Buker, 1962). Such circulating anticoagulants have also been reported in the blood of patients who seemed otherwise to be normal or who had other diseases, notably systemic lupus erythematosus (Margolius, Jackson and Ratnoff, 1961). The acquired circulating anticoagulant described in patients with classical haemophilia has appeared always to inhibit the formation of thromboplastin activity in the early stages of coagulation. This inhibition was accomplished by either inactivating or destroying factor VIII (antihæmophilic factor) (Stefanini and Dameshek, 1955; Biggs and Bidwell, 1959) i.e. the anticoagulant was directed specifically against factor VIII, and this action was illustrated by recent reports (Hall, 1961; Breckenridge and Ratnoff, 1962; Sise, Gauthier, Desforbes and Buker, 1962). A method for the measurement of these circulating anticoagulants has been based on the speed with which the anticoagulant neutralizes factor VIII (Biggs and Bidwell, 1959).

This part of the thesis presents a clinical and laboratory study of a patient with classical haemophilia who developed an unusual form of circulating anticoagulant. The anticoagulant in this case was directed against the production of plasma thromboplastin activity, thus blocking further reaction in the coagulation chain. Factor VIII was not directly antagonized and could in fact be maintained at high hæmostatic level for a period without exhibiting a

a corrective effect on the coagulation mechanism.

### Methods

Blood counts were carried out by standard methods (Dacie, 1956). The Ivy bleeding time, Hess tourniquet test, Lee and White clotting time, clotting time in siliconed tubes, plasma recalcification time, thrombin time, one-stage prothrombin time, prothrombin consumption index, factor VIII assay, thromboplastin generation test, demonstration of circulating anticoagulant by its effect on normal plasma and effects of the anticoagulant on brain thromboplastin were all performed according to the methods of Biggs and Macfarlane (1957). Fibrinogen estimation was done by the Kjeldahl method and euglobulin lysis time by the method described above (p. 47). Plasma proteins were fractionated by precipitation with various concentrations of ammonium sulphate. Each fraction was reconstituted to its original plasma volume with veronal buffer pH 7.5. The percentage prolongation in recalcification time of normal plasma produced by the admixture of 30% reconstituted fraction was arbitrarily taken as relative activity of the anticoagulant in the individual fractions. Precipitin tests on cellulose acetate were performed by an Ouchterlony technique and Grabar immunoelectrophoresis. Antihaemophilic Fraction for transfusion was manufactured and supplied by the Edinburgh and South East Scotland Blood Transfusion Service. It was prepared by ethanol precipitation from fresh plasma and further purified in ethanol glycine by Blombäck's method. Platelet-poor citrated plasma obtained from adding nine volumes of blood obtained by clean venepuncture to one volume 3.8% sodium citrate (B.P.) has been used throughout these tests.

### Clinical Study

The patient, a 40-year old male, was a known haemophilic with a positive

family history. He had suffered from recurrent haematuria since infancy, the attacks occurring primarily twice each winter until more recent years when he had only one episode annually. During many episodes of haematuria the patient had had attacks of renal colic occurring on either side. He had also suffered easy bruising all his life but only had two haemarthroses, both traumatic, resulting in residual damage in the joints, viz. right knee and left ankle. He was able to play football at school and to work as a labourer until 5 years prior to the present investigation. For 10 years before the last admission, haematuria was usually treated by bed rest at home and cleared up rapidly. During an attack of haematuria in 1947 he was admitted to a London Hospital and had an uneventful cystoscopy and retrograde pyelography with minimal blood transfusion cover. On this occasion blood was seen coming from the left ureter and there was mild bilateral hydronephrosis and hydroureter but no evidence of any other local lesion. During the 10 years before the present admission he had largely been treated in hospital for his episodes of haematuria, being transfused with fresh plasma or blood, and controlled rapidly and effectively and without assay of factor VIII content by small amounts given over a short time. He had had no other illnesses of note.

The present episode of haematuria began in February, 1961, settling after one month but recurring five weeks later and resulting in the patient's admission to hospital. He was treated with plasma and blood transfusions without effect and was then transferred to a specialist hospital unit on May 2nd, 1961, where the diagnosis of haemophilia was confirmed; intravenous pyelography done twice in a month showed mild bilateral hydronephrosis. Coliform organisms were cultured repeatedly



from the patient's urine and he was treated with chemotherapy and antibiotics under bacteriological control until finally B. Proteus was isolated. This organism was sensitive only to 'Neomycin' but he was not given this drug. Repeated investigations for Mycobacterium tuberculosis in the urine were negative. The urinary function tests were within normal limits. The patient had osteoarthritic changes consistent with old haemarthrosis in his right knee joint and left ankle. He was repeatedly transfused with fresh blood and plasma without any significant improvement in his clotting time or in his haematuria and it was subsequently found that he had a circulating anticoagulant in his blood and was then transferred to the Royal Infirmary of Edinburgh on July 6th, 1961.

Laboratory investigations here confirmed the diagnosis of haemophilia with a circulating anticoagulant which acted by blocking thromboplastin generation, and not specifically destroying factor VIII. Tomography of the kidneys yielded no further information. Repeated early morning urines and mid-stream urines for bacteriological study were obtained. Intravenous "Pyrexal" followed by colony counts of mid-stream urines at hourly intervals was also done and all these tests proved to be negative. The patient was then given one "unit" human Antihaemophilic Fraction reconstituted in 200 ml. water intravenously every two hours, a unit being the amount prepared from 1,600 ml. fresh A.C.D. plasma. This was repeated six hourly and each Fraction followed immediately by 400 ml. fresh frozen plasma given over the next four hours. He thus received the equivalent of 8,000 ml. fresh plasma per day. By this means the factor VIII level of his blood could be raised to normal but the circulating anticoagulant still largely blocked utilization of that factor

so that the clotting time remained prolonged and the haematuria, although diminished (urinary haemoglobin reduced from 1.16 G.% to less than 0.1 G.%) still continued. It was therefore decided to stop transfusion with Antihaemophilic Fraction and merely maintain the haemoglobin level by transfusions of washed erythrocytes as needed in the hope that by not giving any antihaemophilic factor intravenously the stimulus for the circulating anticoagulant would be removed and its level therefore spontaneously fall. One week later, however, there was no change in anticoagulant titre. The patient was then given oral 'Prednisone' 60 mg. a day with potassium supplement in an endeavour to block the action of the anticoagulant. Unfortunately there was no clinical or laboratory evidence of this happening. Two days later the patient complained of pain in the left hip and was found to have a positive left psoas sign with limitation of movement of the hip joint and hypoaesthesia of the segments supplied by L1 - L4, and weakness of his left quadriceps muscle. It was concluded that he had had a retroperitoneal haematoma into the left psoas sheath and, straight X-ray of the abdomen showed loss of the left psoas shadow. In view of this new bleeding it was decided to give him large doses of porcine Antihaemophilic Fraction in an attempt to overwhelm the blocking anticoagulant. He was given an initial dose the equivalent to 8,000 ml. of fresh plasma to be repeated eight hourly. The laboratory tests, however, whilst showing blood factor VIII levels in excess of 100 per cent following transfusions, showed that the blocking action of the anticoagulant was unaffected and the haematoma rapidly spread retroperitoneally on the left side and extended into the pelvis. The patient required frequent heavy sedation to control the severe abdominal pain and rapidly became oliguric, the urine still being heavily blood stained. He deteriorated

progressively over the next 24 hours and there was clinical evidence of continued extension of the retroperitoneal haematoma which a day later ruptured into his pelvis and resulted in his death.

#### Post-Mortem Examination

At necropsy no lesion in his renal tract was found to account for the haematuria. The findings of bilateral hydronephrosis and hydroureter were confirmed, the latter extending on the right side over the whole length of the ureter and on the left to within one inch of the bladder. Blood was seen only in the right calyceal system though neither kidney showed any gross cause for bleeding. The prostate was normal. There was no evidence of gross infection in the tract. There was a large retroperitoneal haematoma on the left side extending from the psoas sheath upwards into the left hypochondrium then down the lateral wall of the left flank to the pelvis into which it had ruptured. There was no evidence of bleeding elsewhere and the bowel was normal. The lungs showed terminal basal hypostatic pneumonia. The coronary vessels showed fairly marked atheroma. The joints, apart from arthritis in the right knee and left ankle were normal.

On microscopy both kidneys showed moderately severe patchy arterio-sclerotic damage with obliteration of glomeruli, scarring and tubular dilatation. There were scattered haemorrhages throughout the right kidney, both perivascular and intraglomerular. There was no structural abnormality present in the renal tissue to explain the repeated renal haemorrhages. Sections of pelves and ureters revealed no structural abnormality. The myocardium showed no evidence of recent or old infarction. The coronary tree showed a moderate degree of atheromatous damage and there was macroscopic

evidence of severe damage in the interventricular branch of the left coronary artery. There had been a recent haemorrhage into an atheromatous plaque in this area producing a severe degree of stenosis, but not complete obstruction. There was no thrombosis associated with this haemorrhage. Sections of the lungs showed extensive congestion and oedema, particularly in the lower lobes. Liver, spleen, prostate thyroid and adrenals revealed no abnormality.

### Laboratory findings

#### I. General Investigations:-

On admission, the patient's haemoglobin was 11.5 G.%, P.C.V. 35%, M.C.H.C. 32%, leucocyte count 2,800/cu.mm. with a differential count of neutrophils 60%, lymphocytes 35%, monocytes 4% and eosinophils 1%. The E.S.R. was 45 mm. in the first hour (Westergren). The urine contained 1.16 G.% Hb. Serum proteins were 8.5 G.% with electrophoretic fractions of serum albumin 3.6 G.%, alpha 1 globulin 0.2 G.%, alpha 2 globulin 1.0 G.%, beta globulin 1.1 G.% and gamma globulin 2.6 G.%. Serum calcium was 10.7 mg.%, inorganic phosphate 2.4 mg.% and alkaline phosphatase 8 K.A. units. Blood urea nitrogen was 15 mg.%, serum creatinine 1.05 mg.% and serum uric acid 6.5 mg.% (normal = 1-4 mg.%). Urine creatinine was 22 mg.% and the creatinine clearance was 112 ml./minute (normal = 90-120 ml./min.). The urinary calcium output was 208 mg./24 hours on standard ward diet.

#### II. Serological Examination:-

The patient's blood group was A and the probable Rh genotype cDE/cde. The direct Coombs' test was negative and no abnormal antibodies were detected over a temperature range of 4°C. to 37°C. No platelet agglutinins were found in the blood.

#### III. Clotting Studies:-

The following values were obtained - Platelet count 250,000/cu.mm.,



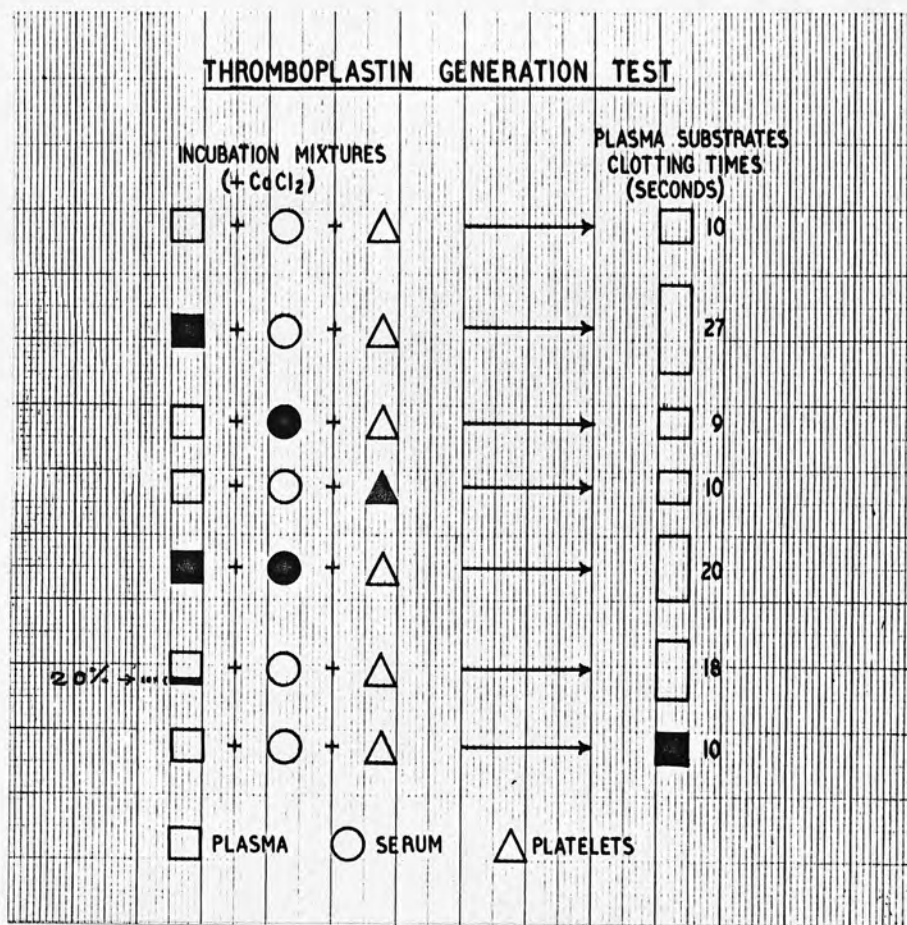


Figure 6. Thromboplastin generation test with clotting times of plasma substrates in seconds showing 1. Normal reaction, 2. Defective reaction of patient's plasma, 3. Normal reaction of patient's serum, 4. Normal reaction of patient's platelets, 5. Defective thromboplastin generation with patient's plasma and patient's serum, 6. Defective reaction with 20% patient's plasma in normal plasma, 7. Normal reaction using the patient's plasma as a fibrinogen substrate. (Clear figures refer to normal blood components and black ones to those of the patient).

bleeding time 2 minutes (normal (N) =  $2\frac{1}{2}$  to 7 minutes), Hess test negative, Lee and White clotting time 56 minutes (N = 5-10 minutes), clotting time in siliconed glass more than two hours (N = 18-25 minutes) and plasma recalcification time between 600 and 1,300 seconds (N = 90-250 seconds). The thrombin time was 13 seconds (control 14 seconds), prothrombin time 14 seconds (control 13 seconds) the prothrombin consumption index 100% (N = 20% or less). The plasma fibrinogen was 330 mg.% (N = 200-500 mg%) and the euglobulin lysis time 238 minutes (N = 120-330 minutes). Assay level of factor VIII in the patient's blood was 18 per cent (N = 60-160 per cent) and assays of factors V, VII and IX were normal. The thromboplastin generation test (Fig. 6) showed that whenever the patient's plasma was used in the system the clotting time was prolonged and that four parts of normal plasma mixed with one part of the patient's plasma did not restore the clotting time of the fibrinogen substrate plasma to normal. With a normal thromboplastin generation mixture, using the patient's plasma as a fibrinogen substrate, normal clotting time was obtained. Both serum and platelets of the patient reacted normally in the thromboplastin generation mixture.

#### IV. Investigations of Circulating Anticoagulant :-

1. Demonstration of the presence of an anticoagulant by its non-specific inhibitory effect on normal blood and plasma.
  - (a) An equal volume of the patient's native plasma (obtained by cold separation without added anticoagulant) added to normal blood in silicone-coated glass tubes prolonged the clotting time of the latter from 21 minutes to 150 minutes.
  - (b) Increasing concentration of the patient's plasma in normal plasma caused progressive marked prolongation of the recalcification time of the latter without

incubation (Table X). The inhibitor was hardly demonstrable when either the normal plasma used in the test or the patient's plasma had been repeatedly frozen and thawed.

2. Test for heparinoid effect. The patient's plasma thrombin time was not shortened by toluidine blue.
3. Effect on acetone dried human brain thromboplastin.
  - (a) The one-stage prothrombin time test was carried out on a normal plasma sample, on the patient's plasma and on a mixture of both plasmas in equal parts. The clotting time of the mixture was not significantly longer than that of the normal plasma.
  - (b) Eight mixtures of 0.1 ml. of patient's plasma and 0.1 ml. of brain thromboplastin suspension were prepared and placed in a water bath at 37°C. At five minutes intervals, one of the mixtures was recalcified with 0.1 ml. M/40 calcium chloride and the recalcification time recorded. The test was repeated using normal plasma. No significant lengthening of the recalcification times following incubation of the patient's plasma over the normal plasma was found.
4. Test for factor VIII destruction.

Factor VIII content of normal plasma was estimated before and after incubation at 37°C. for 30 minutes with an equal volume of the patient's plasma. There was no significant change due to incubation.

#### V. Characterization of the Inhibitory Agent :-

##### 1. Serum inhibitory activity.

The patient's serum showed no antagonistic activity to the

coagulation process in the thromboplastin generation test. Also 30 per cent of barium sulphate adsorbed patient's serum in normal plasma did not alter the recalcification time of the latter compared to the control.

2. Storage and thermal stability.

The patient's plasma stored at  $-20^{\circ}\text{C}$ . preserved its anticoagulant activity for more than 6 weeks. Repeated freezing and thawing of the plasma, however, had a marked deleterious effect on the anticoagulant. A small diminution in the anticoagulant effect was noticed in samples stored at  $4^{\circ}\text{C}$ . for a few weeks. The anticoagulant maintained its potency at room temperature for 48 hours and after heating at  $56^{\circ}\text{C}$ . for 30 minutes, but lost 27 per cent of its potency after heating for 10 minutes at  $72^{\circ}\text{C}$ . At  $80^{\circ}\text{C}$ . it was impossible to demonstrate the anticoagulant because of denaturation of the whole plasma proteins.

3. Effect of organic solvents, ether and chloroform.

Plasma samples separately extracted in the cold with 2 volumes of either solvent for 30 minutes did not lose their anticoagulant power.

4. Effect of inorganic adsorbants.

Aluminium hydroxide and barium sulphate adsorbed plasmas showed no loss of anticoagulant activity.

5. Plasma dialysis.

The patient's plasma was dialyzed against veronal buffer pH 7.5 at  $4^{\circ}\text{C}$ . for 24 hours during which time the buffer was changed 5 times. The anticoagulant activity was minimally



affected.

6. Activity of plasma protein fractions.

The plasma proteins were precipitated by various concentrations of ammonium sulphate. After dialysis of the fractions and reconstitution to the original plasma volume with veronal buffer the inhibitory activity of each fraction was assessed. The anticoagulant was not limited to one fraction (Table XI).

7. Immune antibodies.

No immune antibodies could be detected in the patient's plasma against a purified preparation of Antihaemophilic Fraction, nor against normal or haemophilic plasmas by Grabar immunoelectrophoresis and Ouchterlony precipitin tests.

Discussion

That the patient had classical haemophilia was proved by the clinical and family history and by the low level of factor VIII in his blood. The potent anticoagulant effect of his plasma against normal plasma is demonstrated in Table X, an effect which was obvious even where the patient's plasma was present in as low a concentration as 10 per cent.

The anticoagulant here was neither acting in the fourth stage of clotting i.e. stage of fibrin 'dissolution', nor in the third stage (fibrin formation). The fibrinogen content of the patient's plasma was normal. No increase in spontaneous fibrinolytic activity was demonstrable. The ultimate clot was normal in volume, tensile strength and retractability. That there was no evidence of heparinoid activity was shown by the constantly normal thrombin time both before and after addition of toluidine blue.

The anticoagulant was not acting in the second stage of the clotting mechanism (prothrombin activation stage) as was shown by a normal one-stage Quick prothrombin time. Presumably also the anticoagulant effect was not directed against the activity of the intrinsic or extrinsic thromboplastins, factor V, factor VII or factor X (Stuart Prower factor).

That factor IX (Christmas factor), factor XI (PTA, Rosenthal factor) and factor XII (Hageman factor) were not antagonized was shown by the normality of the serum. The patient's platelets were present in normal numbers and they behaved normally in the thromboplastin generation mixture. The patient's factor VIII blood level was 18% and transfusion with Anti-haemophilic Fraction raised the level by the anticipated amount to within normal limits. This did not, however, rectify the clotting mechanism. Incubation of normal plasma with the patient's plasma did not decrease its factor VIII content. A normal clotting time obtained when the patient's plasma was used as a fibrinogen substrate in the thromboplastin generation test confirmed the lack of antagonism of the anticoagulant to the generated plasma thromboplastin. The site of action of the anticoagulant could therefore only have been in the interaction phase between the various factors necessary for the plasma thromboplastin generation.

The anticoagulant was present in the patient's plasma but was absent from his serum. It was heat stable, only minimally affected by dialysis and not confined to any one salt precipitated plasma protein fraction. It was not soluble in lipid solvents and not adsorbed by inorganic protein adsorbents. It did not appear to be of the nature of a gamma globulin immune antibody. The apparent antagonization of the anticoagulant in the patient's plasma produced by alternatively freezing

and thawing it and the similar effect of normal plasma so treated on the patient's untreated plasma cannot be unequivocally explained; it might be due to platelet contamination and destruction with release of thromboplastin as suggested by Sjölin (1959).

Tocantins' antithromboplastin is claimed to be present in excessive amounts in haemophilic blood. By definition it is an activity existing probably in the form of a lipoprotein in the tissues, blood plasma and serum, directed against the formation and action of thromboplastin. In order to demonstrate it, special techniques have to be used or it is destroyed (Tocantins, 1955). No special precautions were taken in our experiments in that respect and yet the anticoagulant effect still remained. For these various reasons, the anticoagulant met with in this case is considered to be a substance different from the antithromboplastin described by Tocantins.

In some patients sensitization to the Rh - Hr system of blood factors from previous transfusions can give rise to a circulating anticoagulant (Stefanini and Dameshek, 1955). This was not the case in our patient, no antibodies being detected over a wide range of temperature and the direct Coombs' test being negative.

The so-called "Bridge Anticoagulant" (Nour-Eldin and Wilkinson, 1958) has not to our knowledge been characterized. This phenomenon has now been re-evaluated and endowed with a new conception (Serafini, 1959) in that a plasma containing an incomplete thromboplastin such as haemophilic plasma may cause a normal plasma to clot in a shorter time than plasma lacking the same factor because in the former case thromboplastin formation can be completed and further activated by the substrate plasma. Such a phenomenon concerns not only the deficiency of antihæmophilic factor, but even that of the other thromboplastin factors. Indeed it can be proved that, when

thromboplastin is generated with a deficiency of platelet factor, it clots platelet-rich plasma in a shorter time than platelet-poor plasma. This point of view has been also supported by the research of Hougie (1959).

It is difficult to explain this patient's lifelong episodes of haematuria on a haemophilic basis alone in that he had no other spontaneous manifestations of the haemophilic state and only had two haemarthrotic episodes both to trauma during his lifetime. His incapacity was indeed only due to his episodes of haematuria and until the last illness these responded to rest and a short period of transfusion.

Necropsy showed bilateral hydronephrosis and hydroureter of moderate degree and it is hardly conceivable that this was a basic cause of the haematuria. What part, however, it played in his final illness we do not know. Reports on the association of renal lesions with haemophilia are meagre, and the association of even weak circulating anticoagulants with kidney disease accompanied by haemorrhagic tendency seems to be rare (Willoughby and Crouch, 1961).

#### Summary

- (1) A description is given of a circulating anticoagulant that occurred in a patient with classical haemophilia. The anticoagulant blocked the interaction of the thromboplastin generation components but did not destroy factor VIII.
- (2) A clinical study and a laboratory attempt at the characterization of the anticoagulant is presented.

#### Acknowledgements

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TABLE X

Non-specific inhibitory effect of different concentrations of the patient's plasma on the recalcification time of normal plasma.

Percentage of patient's plasma in normal plasma	Zero	10	30	50	70	90	100
Recalcification time in seconds	192	385	564	740	855	1080	1260

TABLE XI

Relative anticoagulant activity of the patient's plasma before and after dialysis, and of ammonium sulphate precipitated fractions of the patient's plasma proteins. The activity was calculated from the per cent prolongation in plasma recalcification time of normal plasma exerted by 30% concentration of the component concerned.

Nature of sample	Patient's plasma	Dialyzed patient's plasma	0 - 25% saturated fraction	25 - 34% saturated fraction	34 - 50% saturated fraction	> 50% saturated fraction
Per cent activity	263	213	157	145	121	154

### Summary

#### I The Fibrinolytic System

A review of the literature is presented together with an evaluation of the present methods for the measurement of its parameters.

The methods adopted in the course of this study include (1) the heated fibrin plate for the estimation of plasmin, (2) the plasma euglobulin lysis time for the determination of the overall "fibrinolytic activity" and/or the activator, (3) a caseinolytic method for the measurement of plasminogen and (4) an assay of antifibrinolysins.

(a) A study of the fibrinolytic system in some congenital and hereditary haemorrhagic disorders was carried out in order to evaluate the theory that, in vivo, the coagulation and the fibrinolytic processes normally exist in a state of dynamic equilibrium. It was thought that if this theory held true, then a change in one of the components of the equilibrium state, (for example a delayed or diminished coagulation mechanism as in certain haemorrhagic disorders) would lead to a balancing diminution in the other component and hence to a decrease in fibrinolytic activity. It was found that the fibrinolytic system was normal in these states and that its activation during and following operative procedures, followed a normal sequence. These findings may cast some doubt on the theory of physiological dynamic haemostasis.

(b) The changes in the fibrinolytic system observed in eighteen patients with cardiac disease undergoing open-heart surgery on cardiopulmonary bypass with hypothermia were studied. This revealed that excessive fibrinolysis is inevitable at some time during the procedure,

but need not, however, cause any serious consequences as shown in the patients studied. Plasminogen and fibrinogen diminution was noted, while changes in the antifibrinolysins were less clear.

## II A Comparison of Haptoglobin Phenotypes in Haemophilics and Normal Persons in Scotland

Haptoglobin phenotyping by starch-gel electrophoresis was done in thirty-eight patients with haemophilia and other congenital and hereditary haemorrhagic disorders, and in one hundred normal individuals in Scotland. This study was undertaken in an endeavour to find a genetic link for these conditions and as a way of investigating the relation between blood groups and/or haptoglobin types and disease states.

Haptoglobin type distribution in these conditions matched that in the control series which, in its turn, was in accord with that of neighbouring populations.

## III Plasma Thromboplastin Generation Blocking Anticoagulant in Haemophilia. Clinico-Pathological Study of a Refractory Case

This investigation presented a clinical study, together with an attempt at the characterization of and speculations on the aetiology of an unusual form of circulating anticoagulant which developed in a patient with classical haemophilia. The anticoagulant in this case blocked the interaction leading to the generation of plasma thromboplastin activity, but did not destroy factor VIII.



PUBLICATIONS

1. Investigations into the fibrinolytic system in certain congenital and hereditary haemorrhagic disorders: A critical evaluation of the theory of dynamic haemostasis. Submitted for publication.
2. Some haematological changes during cardiopulmonary bypass. In preparation.
3. A comparison of haptoglobin phenotypes in haemophilics and normals in Scotland. Vox Sang., in press.
4. Plasma thromboplastin generation blocking anticoagulant in haemophilia. Clinico-pathological study of a refractory case. Am.J.Med., in press.

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